Serum KIF5A, KIF18A, and p53 autoantibodes as Potential Biomarkers of Asbestos Exposure and Disease

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THESIS

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TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
ATSDR	Agency for Toxic Substances and Disease Registry
BRD	Benign Respiratory Disease
CRC	Colorectal Cancer
CV	Coefficient of Variation
DLCO	Diffusing Capacity of the Lungs for Carbon Monoxide
ELISA	Enzyme-Linked Immunosorbent Assay
HCC	Hepatocellular Carcinoma
IARC	International Agency for Research on Cancer
ILO	International Labour Organization
K18Ang	KIF18A (ng/dL)
KIF	Kinesin Family Protein
LLD	Lower Limit of Detection
MDM2	Mouse Double Minute 2 Homolog
MPM	Malignant Pleural Mesothelioma
MSHA	U.S. Mine Safety and Health Administration
NF	Nuclear Factor
NF2	Neurofibromatosis Type 2
NIOSH	U.S. National Institute for Occupational Safety and Health
NSCLC	Non-Small Cell Lung Carcinoma
NTP	U.S. National Toxicology Program
OR	Odds Ratio
OSHA	U.S. Occupational Safety and Health Administration
p53	Tumor Protein 53
p53AAbs	p53 Autoantibodies
PDGF	Platelet-Derived Growth Factor
RNS	Reactive Nitrogen Species
ROC	Receiver Operating Characteristic
ROS	Reactive Oxygen Species
SELDI-TOF	Surface-Enhanced Laser Desorption/Ionization Time-of-Flight
SMRP	Soluble Mesothelin-Related-Peptide
TNF	Tumor Necrosis Factor
TP53	Tumor Protein 53 gene
WHO	World Health Organization

SUMMARY

A set of studies examining three potential biomarkers of asbestos exposure and disease was conducted using stored serum samples from an Italian cohort of 198 asbestos exposed and 164 unexposed workers and a Finnish cohort of asbestosis patients of varying severity (9 ILO Category 0, 43 ILO Category 1, 25 ILO Category 2, and 5 ILO Category 3) who were followed up for cancer incidence (28 asbestos related cancer cases and 51 individuals without asbestos related cancers). Information was collected on demographics, smoking status, asbestos exposure, asbestosis severity, and cancer diagnosis. Enzyme linked immunosorbent assays were run on the stored serum samples to determine the levels of KIF5A, KIF18A, and p53 autoantibodies present.

We found no significant association between p53 autoantibodies and asbestos exposure in this Italian cohort. We also found a non-statistically significant trend of increasing percentages of p53 autoantibody positive individuals in higher ILO categories. We found no association between KIF5A or KIF18A and any form of cancer in this Finnish cohort. We found significantly higher KIF5A levels in asbestos-exposed individuals and significantly lower KIF18A levels in younger and middle-aged, but significantly higher KIF18A levels in older asbestos-exposed individuals in this Italian cohort. We found no significant association between KIF5A and ILO severity, but found increased KIF18A concentrations associated with higher ILO severity scores in this Finnish cohort.

1. INTRODUCTION

Asbestos is a generic name given to a group of six naturally-occurring fibrous silicate minerals (amosite, chrysotile, crocidolite, and the fibrous varieties of tremolite, actinolite, and anthophyllite) that have been widely used in commercial products due to their extraordinary tensile strength, flexibility, poor heat conduction, and relative resistance to chemical attack (ATSDR, 2001; WHO, 2006). Asbestos minerals consist of two groups or classes, serpentine (chrysotile) and amphibole asbestos (amosite, crocidolite, and the fibrous forms of tremolite, actinolite, and anthophyllite), which are differentiated by the arrangement of the silicate crystals.

Asbestos minerals are widespread in the environment as large natural deposits or as contaminants in other minerals. Asbestos is neither volatile nor soluble, but small fibers or clumps may be suspended in air and water. While large fibers are removed by gravitational settling at a size-dependent rate, small fibers may remain suspended for long periods of time (ATSDR, 2001; IARC, 2012). Asbestos fibers can be released into to the air, water, and soil through both natural and anthropogenic processes, with anthropogenic activities being the predominant source of atmospheric asbestos. While environmental releases of asbestos continue to decline in many countries that have stopped mining and phased out its use in most products, its widespread use in the past in industrialized nations represent a legacy source of environmental exposure to both occupational populations and the general population (IARC, 2012; ATSDR, 2001; NTP, 2014). The general population is primarily exposed to low levels of

asbestos via inhalation that present a very low risk to most people. Some people, however may be exposed to higher levels of asbestos if they live near asbestos-containing waste sites or asbestos related industries, if they use asbestos-containing products, or if they live or work in buildings with deteriorating asbestos insulation or that has undergone poorly performed asbestos removal (ATSDR, 2001; NTP, 2014).

While asbestos has been used in small amounts intermittently for thousands of years, modern industrial usage began around 1880 with the development of the Quebec chrysotile fields (IARC, 2012). Over the next 50 years production and use of asbestos increased with a cumulative total of nearly 5000 million kg mined by 1930. At its peak use in the late 1960s and early 1970s asbestos was used in more than 3000 applications or products including roofing, thermal and electrical insulation, cement pipe and sheets, flooring, gaskets, friction materials (e.g. brake pads and shoes), coating and compounds, plastics, textiles, paper, mastics, thread, fiber jointing, and millboard (IARC, 2012; ATSDR, 2001; NTP, 2014). Worldwide production and consumption of asbestos has decreased from its peak in recent decades as health and liability issues became apparent and driven in part by bans on many of its uses in the United States and Western Europe (ATSDR, 2001; NTP, 2014). However, current use of asbestos varies widely internationally as there continues to be substantial production in Russia, Canada, China, Brazil, Zimbabwe, and Kazakhstan and extensive use in South and Central America, Asia, and Africa (ATSDR, 2001; IARC, 2012).

The World Health Organization (WHO) estimated that about 125 million people are exposed to asbestos in the workplace worldwide and at least 107,000 people die each year from

asbestos-related lung cancer, mesothelioma and asbestosis resulting from workplace exposures (WHO, 2014). Historically, workers involved in the mining of asbestos or asbestos-containing minerals, the manufacture of asbestos products, and the construction and shipbuilding industries were potentially exposed to high levels of asbestos (NTP, 2014). Workers who use asbestos insulation, brake repair and maintenance workers, building demolition workers, and asbestos abatement workers are also at risk of exposures to high levels of asbestos (IARC, 2012; ATSDR, 2001; NTP, 2014). In 1990 the U.S. Occupational Safety and Health Administration (OSHA) estimated that around 568,000 workers in production and services industries and 114,000 workers in construction industries may have been exposed to asbestos in the workplace in the U.S. alone (ATSDR, 2001). More recently OSHA has estimated that as many as 1.3 million employees in construction and general industry face significant asbestos exposure while on the job (IARC, 2012). Data analyzed by OSHA and the Mine Safety and Health Administration (MSHA) have shown both reductions in asbestos levels and a changing nature of occupational exposures over the past several decades, transitioning from long-term exposures associated with mining, milling, and product manufacture and fabrication to intermittent short-term exposures during maintenance or building remediation activities (IARC, 2012).

Exposure to asbestos through inhalation can cause a number of conditions including cancer of the lung, larynx, esophagus and ovaries, as well as mesothelioma, asbestosis, and plaques, thickening and effusion in the pleura (ATSDR, 2001; WHO, 2006; WHO, 2014). Positive associations have also been observed between asbestos exposure and cancer of the pharynx, stomach, and colorectum (IARC, 2012). The WHO identified asbestos as one of the most important occupational carcinogens as about half of all occupational cancer deaths are caused

by asbestos (WHO, 2006). While the health effects related to asbestos exposure have primarily been reported for chronic occupational exposures, there is some evidence of these effects occurring following relatively brief occupational exposures of 1-12 months in humans as well as in acute animal studies (ATSDR, 2001). There are a number of case reports and case series that have identified asbestos related disease including asbestosis and mesothelioma in individuals with relatively brief exposure periods, some of whom only had identifiable environmental or domestic exposures (Wagner, 1965; Booth and Weaver, 1986; Ferguson and Watson, 1984; Barbers and Abraham, 1989). However, a number of these cases, especially those with very short periods of identified exposure and/or abnormally short latency periods preceding development of disease have been questioned due to the weak exposure ascertainment and/or highly abnormal disease timeline (Browne and Goffe, 1984; Elmes and Browne, 1986). Other studies of New Jersey and Texas workers involved in the production of amosite-insulated materials, with average exposures of 6-12 months, and of gas-mask factory workers in the UK, with exposures of a few weeks up to 4.5 years, have shown increased incidences of radiographic abnormalities indicative of pulmonary fibrosis and an excess of deaths from respiratory cancers including mesotheliomas (Ehrlich et al., 1992; Levin et al., 1998; Shepherd et al., 1997; Jones et al. 1980; Seidman et al., 1979, 1986). A number of animal studies have also found negative health effects of acute exposures to asbestos in rats, mice, and guinea pigs that have included decreases in antioxidants with concurrent increases in markers of lung injury, early changes in lung structure and localized fibrosis, intense neutrophil alveolitis, progressive fibrosis of the lung, asbestosis, and various lung tumors (Chang et al., 1988; Wagner et al., 1974; Kaiglová et al., 1999; Schoenberger et al., 1982; McGavran et al., 1989; Brody and

Overby, 1989). One additional study found intermediate durations of exposure to asbestos resulted in fibrosis in rats (Donaldson et al., 1988). Despite the findings of negative health effects associated with acute and intermediate exposures to asbestos in some human and animal studies, the Agency for Toxic Substances and Disease Registry (ATSDR) has concluded that further information is needed to understand these potential links and to characterize the underlying dose-response relationship in order to derive a reliable minimal risk level (ATSDR, 2001). The typical long latency periods of up to 20-40 years associated with many of the health effects of asbestos will result in a delayed decrease in the number of asbestos-related diseases and deaths after cessation of exposure, as demonstrated by the burden of asbestos-related diseases still rising in countries that have banned the use of asbestos since the early 1990s (IARC, 2012; WHO, 2006). There is no evidence for a threshold level of exposure – a level of exposure below which there is no risk of an effect occurring – for the carcinogenic effects of asbestos (ATSDR, 2001; WHO, 2006).

Given the large occupational population potentially exposed to asbestos and the severity of associated health effects with no threshold level of exposure, more than 40 countries have banned the use of all forms of asbestos. The WHO has called for globally stopping the use of all types of asbestos as well as the use of proper industrial hygiene preventive measures in work where exposure to asbestos fibers is possible (WHO, 2006). However, the continued production and use of asbestos in many parts of the world, as well as potential exposure to legacy sources of asbestos present a continuing potential for exposure and resultant disease that must still be addressed. The prognosis for fibrotic lung disease (asbestosis), bronchogenic cancer, malignant mesothelioma, other respiratory malignancies, and gastrointestinal cancers is extremely poor.

The identification of biomarkers of exposure and early markers of effect represent a potentially critical tool for the identification of at-risk workers to be targeted with novel health interventions to prevent or provide early treatment to reduce the burden of cancer deaths and disability from asbestos exposure. Current biomarkers to identify or quantify exposure to asbestos fibers include detection and counting of fibers or asbestos bodies in bronchiolar lavage fluid samples, sputum samples, or in autopsied or surgically resected lung tissue samples (ATSDR, 2001). An expert panel convened by ATSDR in 2006 concluded that the most promising biomarker techniques for determining asbestos exposure were analyzing lung tissue from autopsy and determining fiber content from bronchoalveolar lavage fluid, and that while determining fiber content from sputum samples and other blood tests could prove useful in the future, further research was needed (ATSDR, 2006).

Classical methods for detecting health conditions associated with asbestos include imaging such as chest x-ray, computerized tomography, and magnetic resonance imaging as well as quantitative analysis of lung function including changes in pulmonary function and biphasic lung carbon monoxide diffusing capacity (ATSDR, 2001). While these classical measures of effect can be used to identify asbestos-induced health effects, the discovery of biomarkers of early effect for asbestos related cancer may allow for intervention prior to onset or earlier in the process of clinical symptoms developing. A potential intervention to halt or reverse the carcinogenic process induced by asbestos exposure could be the targeting and therapeutic modulation of proteins involved in the genesis and development of tumors (Huszar et al., 2009; Liu et al., 2013; Di Marzo et al., 2014). Potential biomarkers of early effect could include cells or cellular factors present in lung lavage fluid and/or serum of asbestos-exposed individuals that precede

the clinical health effects of asbestos exposure. In its 2001 review ATSDR highlighted several potential biomarkers that were elevated in lung lavage fluid, serum, or urine of asbestos workers compared to unexposed controls that showed promise in early studies, but concluded that further research was needed to develop noninvasive asbestos-specific biomarkers of effect (ATSDR, 2001).

The 2006 expert panel convened by ATSDR reviewed some of the more promising biomarkers of early effect from the literature at the time, specifically blood mesothelin or osteopontin levels, and concluded that while they were very promising, neither biomarker was "ready for primetime" because of unanswered questions regarding their value in predicting the development of mesothelioma due to unacceptable levels of false positives and false negatives (ATSDR, 2006). Initial findings showed that soluble mesothelin-related-protein (SMRP) was a marker of mesothelioma with a sensitivity of 83% and specificity of 95%. In 75% of patients at diagnosis SMRP was elevated and serum levels paralleled clinical course/tumor size (Robinson et al., 2005). Later research found that mean serum SMRP levels were higher in malignant pleural mesothelioma (MPM) patients compared with lung cancer patients, stage 1 MPM SMRP levels were significantly higher than those in asbestos-exposed individuals, and that stage 2-4 SMRP serum levels were significantly higher than those for stage 1 MPM. They reported that SMRP distinguished MPM patients from asbestos-exposed individuals with a sensitivity of 60% and a specificity of 89% (Pass et al., 2008). But another group found no significant difference in mean serum mesothelin levels between malignant mesothelioma cases and controls (Roe et al., 2008).

Early research of another potential biomarker found that elevated serum osteopontin levels were associated with pulmonary plaques and fibrosis compared to plaques alone, fibrosis alone, or normal radiographic findings among asbestos exposed individuals and that serum osteopontin levels were significantly higher in subjects with pleural mesothelioma than in subjects who had exposure to asbestos but who had not developed mesothelioma (Pass et al., 2005). The sensitivity for differentiating mesothelioma patients from asbestos-exposed controls was 77.6% and specificity was 85.5%. However, another study found that osteopontin levels were elevated in subjects with asbestos-related disorders without malignant mesothelioma or lung cancer, which could indicate that serum osteopontin levels may be influenced by nonmalignant processes (Park et al., 2009). In another study serum osteopontin level was higher and could distinguish MPM patients from healthy asbestos-exposed controls, but failed to distinguish MPM from pleural metastatic carcinoma or benign pleural lesions associated with asbestos exposure. The study also found serum mesothelin outperformed osteopontin at distinguishing MPM and that combining the two markers did not improve performance above mesothelin alone, but that both molecules have value as prognostic markers (Grigoriu et al., 2007). Another research group studied whether combining measures of SMRP and plasma osteopontin could increase the sensitivity and specificity in the diagnosis of the epithelioid subtype of MPM. They found significant differences in both SMRP and plasma osteopontin mean levels comparing epithelial MPM patients and healthy subjects or benign respiratory disease (BRD) patients, whereas there was no difference between healthy subjects and BRD patients (Cristaudo et al., 2011). The combination of the two markers into a combined risk index improved both the sensitivity and specificity of epithelial MPM diagnosis. Another

group investigated the performance of serum mesothelin and osteopontin levels, and their combined performance, in distinguishing patients with mesothelioma from those with other non-malignant asbestos-related conditions, as well as whether levels of the biomarkers differed between asbestos exposed subjects and non-exposed controls. This cohort was unique from most others investigating potential biomarkers in that the source of exposure was naturally occurring asbestos in the environment near the subjects' homes. They found that median serum osteopontin and mesothelin levels were significantly higher in mesothelioma patients than in subjects with non-malignant asbestos-related diseases, healthy exposed subjects, and unexposed controls. The sensitivity and specificity of osteopontin in distinguishing mesothelioma from the other groups were 75 and 86%, respectively, while those of mesothelin were 58 and 83%, respectively, and the parallel combination of osteopontin and mesothelin had a sensitivity and specificity of 93 and 73% (Bayram et al., 2014). Ongoing research to discover new biomarkers and into potential panels of biomarkers to improve the sensitivity and specificity of detecting and diagnosing asbestos-related diseases before they have reached later clinical stages is important to advancing efforts to reduce the global burden of asbestos related disease. The present work is an investigation into several potential biomarkers of early effect of asbestos exposure that could potentially be included in such a panel.

2. LITERATURE REVIEW

The WHO estimates that 125 million people are occupationally exposed to asbestos worldwide and OSHA has estimated that as many as 1.3 million workers in construction and general industry face significant exposures to asbestos on the job in the U.S. (WHO, 2014; IARC 2012). Asbestos exposure is known to result in a number of negative health effects, many of which have long latency periods of up to 20-40 years, including cancer. There has been no evidence of a threshold for the carcinogenic effect of asbestos (ATSDR, 2001; IARC, 2012; WHO 2006). The WHO has also raised its estimates of the global burden of asbestos related disease to 107,000 annual deaths primarily from asbestos-related lung cancer, mesothelioma, and asbestosis (WHO, 2014). Given the large population occupationally exposed to asbestos, potential biomarkers of exposure and/or of early effect for disease and cancer risk from asbestos could have a large effect on the burden of disease by identifying individuals at the highest risk for malignancy who could be targeted for more aggressive intervention in the future; at present, such interventions have not yet been defined. Certain members of the kinesin family proteins (KIFs) and p53 autoantibodies may be potential examples of early markers of asbestos related cancer risk.

The long latency period associated with asbestos-induced cancer may allow for the detection of preclinical changes such as genetic and molecular alterations that precede overt disease. These alterations may involve the mutation or amplification of oncogenes, the inactivation of tumor suppressor genes, alteration of pathways involved in resistance to apoptosis, acquired genetic instability, and angiogenesis (IARC, 2012). However, no mutations

in oncogenes or tumor suppressor genes have been directly linked with exposure to asbestos fibers (NIOSH, 2011). *In vitro* studies have shown that asbestos fibers are cytotoxic and clastogenic, but not mutagenic in the Ames assay. It is thought that there may be multiple mechanisms underlying these effects including generation of reactive oxygen (ROS) and nitrogen species (RNS), alterations in mitochondrial function, physical disturbance of cell cycle progression, and activation of several signal transduction pathways (Nymark, 2008). Asbestos has long been recognized to have the potential to induce significant mitotic aberrations leading to chromosomal instability that is associated with cancer. In cell culture experiments asbestos has been shown to induce these chromosomal effects by binding to proteins that regulate the cell cycle, cytoskeleton, and the mitotic process (MacCorkle et al., 2006). These studies showed the importance of the protein binding property of asbestos fibers to the induction of chromosomal abnormalities including defects in chromosome segregation and polyploidy.

In vivo studies have validated some *in vitro* experiments by identifying asbestos-related changes in gene expression such as activation of the nuclear factor (NF)-kB pathway, p53 promoter activation, and cell proliferation induced by tumor necrosis factor (TNF)-alpha and -beta as well as platelet-derived growth factor (PDGF) A and B (Nymark, 2008). Additionally, studies of asbestos-exposed workers' white blood cells have revealed increased levels of sister chromatid exchanges, chromosomal aberrations, and DNA double-strand breaks as well as anti-double-strand DNA antibodies (Fatma et al., 1991; Marczynski et al., 1994). Abnormal p53 protein accumulation as well as increased mutations in the p53 gene have also been detected significantly more often in primary tumor tissue from lung cancer patients exposed to asbestos than in patients with lung cancer who had no known asbestos exposure (Nuorva et al., 1994;

Guinee et al., 1995; Wang et al., 1995; Husgafvel-Pursiainen et al., 1997; Andujar et al., 2013). However, mutations in the p53 gene were not found in tumor tissue samples from a small number of mesothelioma patients with asbestos exposure or in rats with crocidolite-induced mesothelioma (Kitamura et al., 1998; Ni et al., 2000). Another more recent study found polymorphisms in intron 7 of the p53 gene were more frequently identified in asbestos-exposed non-small cell lung carcinoma (NSCLC) and MPM patients than in NSCLC patients without known asbestos exposure and that neurofibromatosis type 2 (NF2) mutations were only detected in MPM patients (Andujar et al., 2013). This second finding agreed with earlier studies that found mutations in the NF2 gene were found in mesothelioma patients, but not lung cancer patients (Sekido et al., 1995; Bianchi et al., 1995). The importance of NF2 mutations and inactivation was confirmed in a knockout mouse model that showed markedly accelerated malignant mesothelioma tumor formation compared to wild-type littermates when exposed to asbestos. Furthermore, loss of NF2 function was observed in half of malignant mesotheliomas from asbestos exposed wild-type mice and p53 gene inactivation was seen in a subset of tumors (Altomare et al., 2005). The spectrum of molecular alterations that occur appears to be different for malignant tumors of the pleural or peritoneal linings than for asbestos-related lung cancers, which may allow for the development of tissue-specific biomarkers of early effect (IARC, 2012).

The kinesin superfamily of proteins (KIFs) currently includes 45 different proteins classified into 14 families (Yu and Feng, 2010). Kinesins are a conserved class of microtubule-dependent molecular motor proteins that have ATPase activity and motion characteristics. The active movement of KIFs supports several critical cellular functions such as mitosis, meiosis, and the

transport of macromolecules, for example, through axonal transport. Different subtypes of KIFs may participate in different cellular functions, but KIF5s and KIF18s primarily participate in mitosis. In mitosis of eukaryotic cells, kinesins participate in spindle formation, chromosome congression and alignment, and cytokinesis. There is a growing body of evidence that altered KIF expression and function may play a role in the development and progression of a number of different human cancers, including in the lung. In their 2010 review, Yu and Feng highlight a number of kinesins overexpressed in several types of cancers and found to be involved in tumorigenesis and metastasis of breast cancer, renal cell carcinoma, nervous system tumors, lung tumors, retinoblastoma tumors, and cervical cancer (Yu and Feng, 2010). Abnormal kinesin expression can alter the equal distribution of genetic materials during cell mitosis due to chromosome hypercondensation, aberrant spindle formation, anaphase bridges, defective cytokinesis, aneuploidy and mitotic arrest (Liu et al., 2013). The resulting loss or gain of genetic material due to the dysfunctional mitotic process can lead to a number of defects in the daughter cells which can promote carcinogenesis and/or the progression of aggressive behavior of the corresponding tumor cells (Mazumdar et al., 2006; Castillo et al., 2007).

It has been shown that KIF18A is essential in the congression of chromosomes and the accurate alignment of the spindle equator and to suppress kinetochore movements during mitosis (Stumpff et al., 2008). It has also been shown that KIF18A is preferentially over-expressed in the majority of tumor cells, but not detectable in most normal tissues except lung and testis. Additionally, KIF18A was positively related to tumor size and clinical tumor-node-metastasis in hepatocellular carcinoma (HCC), tumor grade and metastasis in breast cancer, and tumor stage, lymphatic invasion, lymph node metastasis, venous invasion, and peritoneal

dissemination in colorectal cancer (CRC). In several other studies KIF18A overexpression has been associated with shorter disease free survival and overall survival in HCC patients, poor overall survival in breast cancer patients, and poor overall survival in CRC patients (Shichijo et al., 2005; Liao et al., 2014; Zhang et al., 2010; Nagahara et al., 2011). While less is known about the potential role of KIF5A in cancer, it does show greater than 69% protein homology with its family member KIF5B. KIF5B has been shown to be integral to the survival of several cancer-derived cell lines and is up-regulated in several types of cancer tissues including cancers of the bladder, stomach, skin, and breast, so a possible relationship between KIF5A and cancer is plausible (Yu and Feng, 2010). Additionally it was shown that KIF5B functions as a catalytic subunit of both nuclear factors NF1 and NF2 complex (Hakimi et al., 2002). Given the previously mentioned increase in mutations and inactivation of NF2 found in mesothelioma patients and mouse models, it is plausible that alterations to kinesin-1 family proteins KIF5A and KIF5B expression and/or function may be found in mesotheliomas. Recent studies have also shown that overexpression of KIF5A can contribute to taxane resistance in breast cancer cell lines and breast cancer patients (De et al., 2009; Tan et al., 2012). While studies have not been reported identifying KIF18A and KIF5A in cell culture experiments as specific targets for asbestos interaction or effect, it is plausible that they could be affected by alterations induced by asbestos exposure in the other proteins that regulate mitosis.

The gene encoding p53 (*TP53*) was identified in 1979 and was originally thought to be an oncogene until it was later discovered that researchers had been studying missense mutants of the *TP53* gene instead of the wild-type gene which acts as a tumor suppressor gene (Hofseth et al., 2004). Wild type p53 has multiple functions that result in its tumor suppressor activity by

preventing the propagation of defective cells including involvement in cell-cycle regulation and apoptosis, development, differentiation, gene amplification, DNA recombination, chromosomal segregation, cellular senescence, and DNA repair (Hofseth et al., 2004). Upregulation of p53 occurs in response to a number of cellular stress or damage signals resulting from DNA damage, hypoxia, telomere shortening, and oncogenic stimulation or radiation. This upregulated expression in response to environmental insult can drive the cell into programmed cell death if necessary due to insufficient DNA repair (Hofseth et al., 2004). Disruption of normal p53 function can provide a selective advantage for clonal expansion of pre-neoplastic and neoplastic cells due to escape of cellular arrest and apoptotic controls. The most common cause of p53 inactivation is missense mutations that result in mutant p53 proteins, but inactivation can also occur through proto-oncogene activation, Mdm2 over-expression, dysfunction of cell signaling pathways involved in regulating p53 activity, and nuclear exclusion of p53 resulting in the atypical accumulation of p53 in the cytoplasm rather than the nucleus (Hofseth et al., 2004; Suppiah and Greenman, 2013).

The *TP53* tumor suppressor gene is the most common site identified for genetic mutations in human cancers, which often causes an increase in the stability of mutant p53 protein, leading to its accumulation in cancer cells (Hofseth et al., 2004; Li et al., 2005). These mutations can occur early in the carcinogenic process and often may have a molecular signature based on the type of cancer and exposure linked to that cancer, which could make p53 an attractive biomarker of early effect (Hofseth et al., 2004). In addition to accumulating in cancer cells, mutant p53 protein has also been found to accumulate in pre-neoplastic lesions and in normal tissues surrounding the tumors, which can lead to correspondingly high levels of mutant p53 in

extracellular fluids such as serum (Mattioni et al., 2013; Hemminki et al., 1996). The inactive mutant p53 protein has the potential to bind and form complexes with wild type p53 protein, which both inactivates the functional wild type p53 proteins and prolongs their half-life by stabilizing their normally rapid degradation (Cordes et al., 2009; Hemminki et al., 1996). The inactivation of p53 proteins and accumulation of both mutant and wild type p53 proteins in the tissue and serum can lead to the production of p-53 autoantibodies, and in fact there is a close correlation between serum p-53 autoantibodies and p53 overexpression in corresponding tissues (Mattioni et al., 2013; Mattioni et al., 2015). It seems p53 mutation alone is not sufficient to induce autoantibody formation as it has been shown that only 20-50% of patients with detectable p53 mutations produce detectable autoantibodies, but instead it may be the elevated levels of p53 protein present in the nucleus and cytoplasm that spills over into plasma and triggers autoantibody production (Suppiah and Greenman, 2013; Soussi, 2000). However, there is generally very good correlation between the presence of p53 autoantibodies and TP53 mutations or accumulations of mutant p53 protein in tumor tissue, and p53 autoantibodies have been detected in the sera of patients with most types of cancer (Soussi, 2000; Li et al., 2005).

Serum p53 autoantibodies have been found in patients with a number of pre-malignant diseases and cancers including Barrett's metaplasia of the esophagus during transition from low to high grade dysplasia. Additionally, serum p53 autoantibodies have been found in people at elevated risk for cancer, such as ulcerative colitis patients at high risk of colon cancer, heavy smokers and individuals with chronic obstructive pulmonary disease at high risk of lung and other cancers, and in workers exposed to occupational carcinogens including asbestos before

any clinical evidence of malignancy. Serum p53 autoantibodies have also been found in a number of cancer patient populations including those with breast and bronchial carcinomas, bladder and colorectal cancers, advanced serous ovarian cancer, and head and neck cancers (Cordes et al., 2009; Anderson et al., 2010; Mattioni et al., 2013; Suppiah and Greenman, 2013; Li et al., 2005). The fact that p53 autoantibodies are detectable in individuals prior to the development or clinical diagnosis of malignant disease with reported lead times to diagnosis ranging anywhere from less than 1 year to 12 years suggests they may possess predictive value for subsequent development of cancer (Li et al., 2005; Mattioni et al., 2013; Pedersen et al., 2013). It has also been noted in several different cancer patient populations that the occurrence of p53 autoantibodies is associated with poorer prognosis than for patients without detectable serum p53 autoantibodies (Cordes et al., 2009). In a number of recent studies of ovarian cancer patients the presence of p53 autoantibodies was either not associated with prognosis or was associated with a modestly more favorable prognosis, and other recent analyses of cancer patient populations show no independent prognostic value of p53 autoantibodies (Anderson et al., 2010; Suppiah and Greenman, 2013). The reported sensitivity of p53 autoantibodies as a tumor marker in cancer patients has generally been low, while the specificity as a tumor marker has been high, but because p53 autoantibodies have been found in numerous cancer types, the presence of p53 autoantibodies are not a specific marker for a particular cancer (Cordes et al., 2009; Anderson et al., 2010; Suppiah and Greenman, 2013). One potential solution to the low sensitivity of p53 autoantibodies as a marker of tumor presence, and to improve the specificity for type of cancer, is to include them in a panel with other biomarkers to improve the combined diagnostic potential (Anderson et al., 2010;

Pedersen et al., 2013). Additionally, p53 autoantibodies may still be useful as a tool to build a risk stratification score to identify individuals at highest risk of developing cancer for increased surveillance and/or preventive interventions (Pedersen et al., 2013). Another potential limitation of p53 autoantibodies is the observation that serum levels appear to correlate with smoking status, increasing from non-smokers to ex-smokers and current smokers, with heavy smokers having the highest prevalence (Li et al., 1999; Mattioni et al., 2013). This highlights the importance of having accurate information on smoking status when assessing the relationship between p53 autoantibody levels and risk of cancer.

Prior studies examining biomarkers in this cohort of Finnish asbestosis cases, originally recruited in 1978-79 at the Finnish Institute of Occupational Health in Helsinki, have targeted analysis of a number of cancer related biomarkers including p53 autoantibodies (Brandt-Rauf et al., 1992; Partanen et al., 1994a, 1994b, 1995; Hemminki et al., 1996; Husgafvel-Pursiainen et al., 1997; Li et al., 2005). This cohort, described in more detail below, had blood samples collected as part of annual visits between March 1980 and August 1987 and were followed up as to health status through the end of 2007. The various biomarkers that have been studied were detectable in banked serum samples years prior to diagnosis of cancer in some cases and the combination of all of the identified biomarkers yielded high specificity (0.85), positive predictive value (0.76), and moderate negative predictive value (0.66), but the sensitivity for the combined biomarkers remained less robust (0.51), thus limiting their potential clinical application (Li et al., 2005). The discovery of additional biomarkers with improved sensitivity for the subsequent development of cancer is important for clinical applications to have the potential of reducing the burden of asbestos related cancer.

Recent examination of a subset of the stored serum samples was undertaken using a proteomic approach based on surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry, an approach which has shown promise for the discovery of new biomarkers of cancer risk (Tooker et al., 2011; Diamandis, 2004). The approach was applied to single serum samples for 35 asbestos workers who subsequently developed cancers (4 malignant mesotheliomas and 31 lung cancers) and 35 asbestos workers who did not develop cancer, group matched for age, gender, race, and smoking status. The sample analyzed for each worker was that closest to, but prior to, the diagnosis of cancer for the 35 cases and the most recent sample for the 35 non-cancer controls. The average time interval between sample collection and the subsequent diagnosis of cancer was 3.7 years (range=1-14 years). The results identified three protein peaks that could predict the development of cancer with good sensitivity (0.87) and specificity (0.70). While one minor peak did not correspond to any known protein, the other two protein peaks were identifiable by protein isolation, digestion, and sequencing analyses and correspond to two members of the kinesin superfamily of proteins, KIF5A and KIF18A (Tooker et al., 2011). As explored above, both of these proteins have been suspected to be involved in the carcinogenic process and could serve as potential biomarkers for risk of disease (Yu and Feng, 2010). The possible combination of KIF5A and KIF18A along with other identified biomarkers of cancer risk such as p53 autoantibodies may allow for a panel of biomarkers with sufficient sensitivity, specificity, and positive and negative predictive values to allow for clinical use in identifying individuals with asbestos exposure at highest risk of cancer and targeting those individuals for potential interventions to delay or reverse the disease process.

3. METHODS

3.1 Study Subjects and Samples

3.1.1 Finnish Cohort

In 1978-79 a cohort of 259 pneumoconiosis patients was assembled at the Finnish Institute of Occupational Health in Helsinki (Brandt-Rauf et al., 1992). These were all Finnish workers with compensable asbestosis or silicosis who were referred to the Institute for further evaluation and who fulfilled the usual diagnostic criteria for their disease. The cohort included 115 cases of asbestosis and 144 cases of silicosis who were planned to be followed prospectively for at least ten years to evaluate the course of their disease. The baseline evaluations in 1978-79 included complete medical histories, including demographic data and occupational and smoking histories, physical examinations, spirometry and chest radiographs. On annual visits between March 1980 and August 1987 blood samples were also requested of some of the participants (particularly the asbestosis cases), and for those participants who consented, blood samples were collected by routine venipuncture techniques and 2 ml aliquots of serum were separated and stored frozen at -70^{o}C. For various reasons, some cases were lost to follow-up, failed to show up for every scheduled appointment or refused to give blood samples. Excluding these, the cohort consists of those 110 cases of asbestosis with at least one available stored serum samples and follow-up as to health status through the end of 2007. The cohort is thus composed of 110 Finnish workers with asbestosis, 102 of whom are male (93%) and 8 of whom are female (7%). The average age of the subjects at the end of sample collection in 1988 was 66.8 years with a range of 40-89 years (2 in their 40s; 22 in their 50s; 42

in their 60s; 33 in their 70s; 11 in their 80s). Most cases had many years of estimated exposure to asbestos (average=20 years; range=2-44 years) in job categories with high likelihood of asbestos exposure (asbestos insulator - 30%; asbestos miner - 24%; asbestos cement worker - 19%; asbestos sprayer - 10%; other miscellaneous asbestos worker - 17%). As a result, estimated exposures were relatively high with an average estimated cumulative exposure of 523 fiber-years/mL (range=14-1750 fiber-years/mL). The cohort includes 27% non-smokers, 44% ex-smokers, and 29% current smokers (58% of whom average 1-14 cigarettes/day, 38% of whom average 15-24 cigarettes/day, and 4% of whom average more than 24 cigarettes/day). It should be noted that the vast majority of subjects in this study are white males. This is due to the fact that in the asbestos industry in Finland until very recently, these jobs were almost exclusively occupied by white males.

Cancer incidence through December 31, 2007 was determined using the Finnish Cancer Registry, a national registry with a complete coverage of diagnosed cancers in the country. At that time, 55 of the 110 asbestosis cases had developed malignant tumors including 34 lung cancers (10 squamous cell carcinomas, 5 adenocarcinomas, 6 small cell carcinomas, 2 bronchoalveolar carcinomas, 7 anaplastic carcinomas, and 4 lung cancers that were not further specified as to type), 4 malignant pleural mesotheliomas, and 17 other cancer of various types (larynx, colorectum, esophageal, urinary bladder, kidney, pancreas, prostate, brain, gallbladder, melanoma and lymphoma). The remaining 55 asbestosis cases in the cohort were followed up through the Finnish Institute of Occupational Health and confirmed to have not developed any malignant tumor as of the end of 2007 and either be still living (13) or having died of other causes (42).

As noted, all 110 patients in this cohort had at least one serum sample collected between 1980 and 1987 available for ELISA analysis. All serum samples have been kept frozen at -70°C since the time of collection and have been randomly recoded so that the analyses were performed blinded to subject identity and case/control status. For the 55 asbestosis cases without cancer there are 196 serum samples available ranging from 1-7 per subject (10 subjects with 1 sample, 9 subjects with 2 samples, 6 subjects with 3 samples, 13 subjects with 4 samples, 8 subjects with 5 samples, 5 subjects with 6 samples, and 4 subjects with 7 samples), and for the 55 asbestosis cases with cancer there are 168 serum samples available ranging from 1-7 per subject (15 subjects with 1 sample, 9 subjects with 2 samples, 8 subjects with 3 samples, 11 subjects with 4 samples, 6 subjects with 5 samples, 5 subjects with 6 samples, and 1 subject with 7 samples), all collected prior to the date of cancer diagnosis which ranges from 1981 to 2007.

3.1.2 Italian Cohort

Information and samples from asbestos-exposed workers and unexposed controls were provided by the University of Perugia Occupational/Environmental Medicine Clinic for the years 2007-2011. The asbestos-exposed workers were recruited by the Italian National Institute for Insurance against Accidents at Work (Istituto Nazionale per L'Assicurazione contro gli Infortuni sul Lavoro - INAIL). For these workers information was collected on age, gender, years of asbestos exposure, and smoking history. They were evaluated for asbestos-related disease by chest radiography, spirometry and diffusion capacity (DLCO), and 198 of the workers were deemed to not have asbestosis. These patients had the following characteristics: average age =

62 with a range = 36-83 (1 in his 30s; 6 in their 40s; 52 in their 50s; 94 in their 60s; 38 in their 70s; 8 in their 80s); all white males; average asbestos exposure = 26 years (range = 11-38 years); 32% non-smokers, 49% ex-smokers, and 19% current smokers (52% of whom average 1-14 cigarettes/day, 40% of whom average 15-24 cigarettes/day, and 8% of whom average more than 24 cigarettes/day). During the same period, normal, healthy, asbestos unexposed workers were recruited at the University of Perugia hospital and out-patient clinics and 164 were selected as controls based on similar age, gender, and demographic area. Information was also collected on their occupation and smoking history. Based upon occupational histories all were deemed to have had no likely exposure to asbestos or other workplace carcinogens. They had the following characteristics: average age = 57 with a range = 21-99 (7 in their 20s; 13 in their 30s; 35 in their 40s; 51 in their 50s; 13 in their 60s; 15 in their 70s; 23 in their 80s; 3 in their 90s); all white males; 34% non-smokers, 37% ex-smokers, and 29% current smokers (48% of whom average 1-14 cigarettes/day, 35% of whom average 15-24 cigarettes/day, and 17% of whom average more than 24 cigarettes/day).

3.2 Laboratory Procedures

The laboratory procedures described below were used to analyze serum samples from both the Finnish and Italian cohorts.

3.2.1 Kinesin Procedures

Serum samples were thawed and analyzed for the presence of KIF5A and KIF18A proteins by commercially available ELISAs. In both cases, the assays are quantitative sandwich ELISAs utilizing microtiter plates pre-coated with a monoclonal antibody specific for the particular KIF.

After incubation of the sample, a biotin-conjugated polyclonal antibody specific for the particular KIF is added followed by avidin-conjugated horseradish peroxidase and 3,3',5,5'-tetramethylbenzidine substrate solution. The color change is measured spectrophotometrically at 450 nm and is converted into the concentration of KIF in the sample by comparison to a standard curve generated from known concentrations of purified KIF protein (run in duplicate) on each plate. The KIF concentration of each sample/individual was then compared to a cutoff level to determine positive or negative status for altered protein expression. The optimal cutoff level for KIF5A and KIF18A protein expression was explored via several methods in each cohort including: the mean KIF5A level among the unexposed individuals plus 2 standard deviations; the mean KIF18A level among the unexposed individuals minus 1 standard deviation, due to a large standard deviation (greater than half the mean value which would result in a negative concentration) among unexposed KIF18A levels; the mean KIF18A level among the unexposed individuals plus 1 standard deviation; and empirically determined cutoffs utilizing receiver operating characteristic (ROC) analyses. The direction of the cutoffs explored is due to the observed increased expression in KIF5A levels and decreased expression in KIF18A levels observed in a subset of asbestos cancer patients from the same Finnish cohort reported previously, as well as the observed increases in KIF18A expression reported in the literature above (Shichijo et al., 2005; Liao et al., 2014; Zhang et al., 2010; Nagahara et al., 2011; Tooker et al., 2011). These assays have been demonstrated to be highly reproducible and to have high sensitivity (LLD<118 pg/mL) and specificity (no cross-reactivity between each specific KIF and known analogues). The manufacturer reported intra-assay coefficient of variation is <10% and inter-assay coefficient of variation is <12%. Finally, from the ELISA results comparisons were

made between the KIF levels found in serum and exposure to asbestos or the subsequent development of cancer, as described below; this analysis was applied to all cancers in the cohort as well to cancers most directly related to asbestos exposure, such as lung cancers and mesotheliomas.

3.2.2 p53 Autoantibody Procedures

Serum samples were thawed and analyzed for the presence and concentration of p53 autoantibodies by commercially available ELISAs. The quantitative assay utilizes microtiter plates pre-coated with recombinant human wild-type p53 protein. After incubation of the sample diluted 1:100, the plate is washed to remove any unbound material and a horseradish peroxidase-conjugated purified goat anti-human polyclonal antibody is added to the wells which binds to any captured human p53 antibody. Following incubation and a wash step a chromogenic substrate is added to the wells. The horseradish peroxidase catalyses the conversion of the chromogenic substrate 3,3',5,5'-tetramethylbenzidine from a colorless solution to a blue solution (or yellow after the addition of hydrochloric acid stopping reagent), the intensity of which is proportional to the amount of human p53 antibody in the test sample. The color change is measured spectrophotometrically at 450 nm and is converted into the concentration of p53 antibody in the sample by comparison to a standard curve generated from known concentrations of purified anti-human p53 antibody (run in duplicate) on each plate. The p53 antibody concentration of each sample was then compared to a cutoff level calculated from the standard curve on each plate to determine positive or negative status for p53

autoantibody expression. Finally, from the ELISA results comparisons were made between the p53 autoantibody levels found in serum and asbestos exposure and severity of asbestosis.

3.3 Data Analysis

3.3.1 Finnish Cohort

Both KIF5A and KIF18A status (Positive/Negative) were assessed as predictors of cancer diagnosis (Yes/No) and along with p53 autoantibody status (Positive/Negative) as predictors of ILO International Classification of Radiographs of Pneumoconioses severity scores in separate logistic regression models using generalized estimating equations (SAS Proc Genmod), which take into account the correlated nature of the responses from repeated measures of the subjects. Models were run to assess the relationship for all cancers as well as for cancers most directly related to asbestos exposure, such as lung cancers and mesotheliomas. Each model additionally explored the potential impact of age, gender, smoking status (current/former/never), and cumulative asbestos exposure (fiber-years/cubic meter).

3.3.2 Italian Cohort

Autoantibody status (Positive/Negative) for KIF5A, KIF18A, and p53 were each assessed as the outcome in separate logistic regression models (SAS Proc Logistic) based upon each subject's status of asbestos exposure (Exposed/Unexposed) and alternatively based upon each subject's cumulative asbestos exposure (years). In each logistic regression model, we additionally explored the potential impact of age and smoking status (current/former/never).

4. SERUM KIF5A, KIF18A, AND p53 AUTOANTIBODY CONCENTRATIONS AS POTENTIAL BIOMARKERS OF ASBESOTS EXPOSURE

4.1 Background

The WHO estimates that 125 million people are occupationally exposed to asbestos worldwide and OSHA has estimated that as many as 1.3 million workers in construction and general industry face significant exposures to asbestos on the job in the U.S. (WHO, 2014; IARC 2012). Asbestos exposure is known to result in a number of negative health effects, many of which have long latency periods of up to 20-40 years, including cancer and there has been no evidence of a threshold for the carcinogenic effect of asbestos (ATSDR, 2001; IARC, 2012; WHO 2006). The WHO has also raised its estimates of the global burden of asbestos related disease to 107,000 annual deaths primarily from asbestos related lung cancer, mesothelioma, and asbestosis (WHO, 2014). Given the large population occupationally exposed to asbestos, potential biomarkers of exposure and/or of early effect for cancer risk from asbestos could have a large effect on the burden of disease by identifying individuals at the highest risk for malignancy who could be targeted for more aggressive intervention. Certain members of the kinesin family proteins (KIFs) and p53 autoantibodies may be potential examples of markers of asbestos exposure and/or related cancer risk.

The kinesin superfamily of proteins (KIFs) currently includes 45 different proteins classified into 14 families (Yu and Feng, 2010). Kinesins are a conserved class of microtubule-dependent molecular motor proteins that have ATPase activity and motion characteristics. Different subtypes of KIFs may participate in different cellular functions, but KIF5s and KIF18s primarily

participate in mitosis. While studies have not been reported identifying KIF18A and KIF5A in cell culture experiments as specific targets for asbestos interaction or effect, it is plausible that they could be affected by alterations induced by asbestos exposure in the other proteins that regulate mitosis. Abnormal kinesin expression can alter the equal distribution of genetic materials between daughter cells during cell mitosis. This may occur due to chromosome hypercondensation, aberrant spindle formation, anaphase bridges, defective cytokinesis, aneuploidy and mitotic arrest (Liu et al., 2013). The resulting loss or gain of genetic material due to the dysfunctional mitotic process can lead to a number of defects in the daughter cells which can promote carcinogenesis and/or the progression of aggressive behavior of the corresponding tumor cells (Mazumdar et al., 2006; Castillo et al., 2007).

The *TP53* tumor suppressor gene is the most common site identified for genetic mutations in human cancers, which often causes an increase in the stability of mutant p53 protein, leading to its accumulation in cancer cells (Hofseth et al., 2004; Li et al., 2005). These mutations can occur early in the carcinogenic process and often may have a molecular signature based on the type of cancer and exposure linked to that cancer, which could make p53 an attractive biomarker of early effect (Hofseth et al., 2004). The inactivation of p53 proteins and accumulation of both mutant and wild type p53 proteins in the tissue and serum can lead to the production of p53 autoantibodies, and in fact there is a close correlation between serum p53 autoantibodies and p53 overexpression in corresponding tissues (Mattioni et al., 2013; Mattioni et al., 2015). Serum p53 autoantibodies have been found in patients with a number of pre-malignant diseases and cancers and in workers exposed to occupational carcinogens including

asbestos before any clinical evidence of malignancy (Cordes et al., 2009; Anderson et al., 2010; Mattioni et al., 2013; Suppiah and Greenman, 2013; Li et al., 2005).

This study examines the potential relationship of asbestos exposure to alterations in KIF5A and KIF18A serum concentrations and p53 autoantibody serum concentrations to determine if they may be potential biomarkers of asbestos exposure and increased risk of subsequent asbestos-related cancers.

4.2 Materials and Methods

4.2.1 Cohort

Information and samples from asbestos-exposed workers and unexposed controls were provided by the University of Perugia Occupational/Environmental Medicine Clinic for the years 2007-2011. The asbestos-exposed workers were recruited by the Italian National Institute for Insurance against Accidents at Work (Istituto Nazionale per L'Assicurazione contro gli Infortuni sul Lavoro - INAIL). Information was collected on age, gender, years of asbestos exposure, and smoking history of these workers. They were evaluated for asbestos-related disease by chest radiography, spirometry and diffusion capacity (DLCO), and 198 of the workers were deemed to not have asbestosis. These patients had the following characteristics: average age = 62 with a range = 36-83 (1 in his 30s; 6 in their 40s; 52 in their 50s; 94 in their 60s; 38 in their 70s; 8 in their 80s); all white males; average asbestos exposure = 26 years (range = 11-38 years); 32% non-smokers, 49% ex-smokers, and 19% current smokers (52% of whom average 1-14 cigarettes/day, 40% of whom average 15-24 cigarettes/day, and 8% of whom average more than 24 cigarettes/day). During the same period, normal, healthy, workers without known

asbestos exposure were recruited at the University of Perugia hospital and out-patient clinics and 164 were selected as controls based on similar age, gender, and demographic area. Information was also collected on their occupation and smoking history. Based upon occupational histories all were deemed to have had no likely exposure to asbestos or other workplace carcinogens. They had the following characteristics: average age = 57 with a range = 21-99 (7 in their 20s; 13 in their 30s; 35 in their 40s; 51 in their 50s; 13 in their 60s; 15 in their 70s; 23 in their 80s; 3 in their 90s); all white males; 34% non-smokers, 37% ex-smokers, and 29% current smokers (48% of whom average 1-14 cigarettes/day, 35% of whom average 15-24 cigarettes/day, and 17% of whom average more than 24 cigarettes/day).

4.2.2 Laboratory Procedures

Serum samples were thawed and analyzed for the presence of KIF5A and KIF18A proteins at the University of Illinois at Chicago by commercially available ELISAs in 2013. In both cases, the assays are quantitative sandwich ELISAs utilizing microtiter plates pre-coated with a monoclonal antibody specific for the particular KIF. After incubation of the sample, a biotin-conjugated polyclonal antibody specific for the particular KIF is added followed by avidin-conjugated horseradish peroxidase and 3,3’,5,5’-tetramethylbenzidine substrate solution. The color change is measured spectrophotometrically at 450 nm and is converted into the concentration of KIF in the sample by comparison to a standard curve generated from known concentrations of purified KIF protein (run in duplicate) on each plate. The KIF concentration of each sample/individual was then compared to a cutoff level to determine positive or negative status for altered protein concentrations. The optimal cutoff level for KIF5A and KIF18A protein

concentrations was explored via several methods including: the mean KIF5A level among the unexposed individuals plus 2 standard deviations; the mean KIF18A level among the unexposed individuals minus 1 standard deviation, due to a large standard deviation (greater than half the mean value which would result in a negative concentration) among control KIF18A levels; the mean KIF18A level among the unexposed individuals plus 1 standard deviation; and empirically determined cutoffs utilizing ROC analyses. The direction of the cutoffs explored is due to the observed increase in serum KIF5A levels and decreased serum KIF18A levels observed in a subset of asbestos cancer patients from an occupationally exposed Finnish asbestosis cohort reported previously, as well as the observed increases in KIF18A expression reported in the literature above (Shichijo et al., 2005; Liao et al., 2014; Zhang et al., 2010; Nagahara et al., 2011; Tooker et al., 2011). These assays have been demonstrated to be highly reproducible and to have high sensitivity (LLD<118 pg/mL) and specificity (no cross-reactivity between each specific KIF and known analogues). The manufacturer reported intra-assay coefficient of variation (CV) is <10% and inter-assay CV is <12%. We were unable to calculate intra-assay CV, but our calculated inter-assay CV was 31.74%. Finally, from the ELISA results comparisons can be made between the KIF levels found in serum and exposure to asbestos as described below.

Serum samples were thawed and analyzed for the presence and concentration of p53 autoantibodies at the University of Illinois at Chicago by commercially available ELISAs in 2012. The quantitative assay utilizes microtiter plates pre-coated with recombinant human wild-type p53 protein. After incubation of the sample diluted 1:100, the plate is washed to remove any unbound material and a horseradish peroxidase-conjugated purified goat anti-human polyclonal antibody is added to the wells which binds to any captured human p53 antibody.

Following incubation and a wash step a chromogenic substrate is added to the wells. The horseradish peroxidase catalyses the conversion of the chromogenic substrate 3,3',5,5'-tetramethylbenzidine from a colorless solution to a blue solution (or yellow after the addition of hydrochloric acid stopping reagent), the intensity of which is proportional to the amount of human p53 antibody in the test sample. The color change is measured spectrophotometrically at 450 nm and is converted into the concentration of p53 antibody in the sample by comparison to a standard curve generated from known concentrations of purified anti-human p53 antibody (run in duplicate) on each plate. The p53 antibody concentration of each sample was then compared to a cutoff level calculated from the standard curve on each plate to determine positive or negative status for p53 autoantibody expression. The calculated Intra-Assay CV was 6.87% and the calculated Inter-Assay CV was 22%. Finally, from the ELISA results comparisons can be made between the p53 autoantibody levels found in serum and asbestos exposure.

4.2.3 Data Analysis

Distributions of KIF5A, KIF18A, and p53 autoantibody levels (continuous) were evaluated for normality and Spearman correlations were assessed between the continuous biomarkers, continuous exposure (years) and age. Individuals with missing variables were dropped from the analysis (n=4 missing age; n=26 missing smoking status; n=4 missing exposure years; n=27 total missing (7.5%)). Mean levels of each potential biomarker were compared in relation to asbestos exposure via parametric and non-parametric analyses where appropriate as well as via receiver operating characteristic (ROC) analysis. Receiver operating characteristic analysis is a method of analyzing the predictive or discriminatory performance of a potential biomarker or diagnostic

test by plotting the sensitivity (true positive rate) against 1-specificity (the false positive rate) for a range of potential cut-points. From this plot one can determine the optimal cut point for each biomarker to differentiate between a binary outcome, in this case exposed and unexposed individuals, utilizing various methods including Youden's statistic and Euclidian distance from the perfect classifier (point 0, 1) (Youden, 1950). Univariate logistic regression models for each biomarker on exposure status were run for the ROC analysis. Multivariable logistic regression models were also run including all three biomarkers and the covariates of age and smoking status as well as interaction terms for each covariate and the potential biomarkers of interest to explore their association with exposure status. Additionally, KIF5A, KIF18A, and p53 autoantibody status (Positive/Negative) were each assessed as the outcome in separate logistic regression models (SAS Proc Logistic) based upon each subject's status of asbestos exposure (Exposed/Unexposed) or alternatively based upon each subject's duration of asbestos exposure (years). In each logistic regression model, we additionally explored the effects of age, smoking status (current/former/never), and the other potential biomarkers.

4.3 Results

General descriptive statistics on the cohort including biomarker expression levels, asbestos exposure years, and the measured covariates of age and smoking status are presented in Table I. Autoantibodies for KIF5A, KIF18A, and p53 were found to be log-normally distributed by statistical tests for normality including the Shapiro-Wilk ($p<0.0001$), Kolmogorov-Smirnov ($p<0.01$) and Anderson-Darling ($p<0.005$). Therefore potential differences in serum biomarker levels between exposed and unexposed individuals were assessed on log-transformed

biomarker values and via the non-parametric Wilcoxon rank-sum test. Exposed individuals were significantly older than control individuals (p<0.0001) and smoking status was also different between exposed and unexposed individuals (p=0.0335) (Table I). Exposed individuals had significantly higher mean levels of KIF5A (p<0.0001), significantly lower mean levels of KIF18A (p=0.0008), but no difference in mean levels of p53 autoantibodies (p=0.766) in serum compared to the unexposed individuals in non-parametric Wilcoxon bivariate analyses. ANOVA results showed that smoking status was not significantly associated with KIF5A (p=0.6404) or p53 autoantibody serum concentrations (p=0.8294), but was associated with KIF18A serum concentrations (p=0.0386), with lower KIF18A concentrations in ex-smokers than current smokers, and age (p=0.0002), with ex-smokers significantly older than current or never smokers.

TABLE I - Serum KIF5A, KIF18A, p53 Autoantibodies, Asbestos Exposure, Age and Smoking Status of Italian Workers[a]

	Exposed (n=198)	**Unexposed (n=164)**	[b]
Age (years)	63.2 (8.6)	57.2 (17.0)	(p<0.0001)*
KIF5A (ng/mL)	2.01 (2.1)	1.02 (1.29)	(p<0.0001)*
KIF18A (ng/mL)	356.39 (157.57)	470.13 (275.4)	(p=0.0008)*
p53AAbs (U)	0.04 (0.1)	0.03 (0.09)	(p=0.766)
Asbestos exposure (years)	25.96 (6.13)	0 (0)	
Smoking			(p=0.0335)*
Current	37 (18.69%)	42 (25.61%)	
Former	95 (47.98%)	52 (31.71%)	
Never	62 (31.31%)	48 (29.27%)	
Missing	4 (2.02%)	22 (13.41%)	

[a] Age, KIF5A, KIF18A, p53AAbs, and Asbestos exposure are represented as: mean (standard deviation); Smoking Status represented as: n (%).

[b] p-values from Student's T-test for Age, Wilcoxon rank-sum test for KIF5A, KIF18A, p53AAbs, and Chi squared test for smoking.
* indicates significant p<0.05

As shown in Table II, age was significantly inversely associated with continuous KIF5A serum concentrations (rho=-0.11, p=0.0371), and with continuous KIF18A concentrations (rho= -0.29, p<0.0001), and significantly positively correlated with continuous p53 autoantibody concentrations (rho=0.38, p<0.0001), and with asbestos exposure years (rho=0.33, p<0.0001). Serum KIF5A and KIF18A were not significantly correlated (p=0.1412), but both KIF5A (rho= -0.16, p=0.0028) and KIF18A (rho= -0.11, p=0.0313) were significantly inversely correlated with p53 autoantibody concentrations.

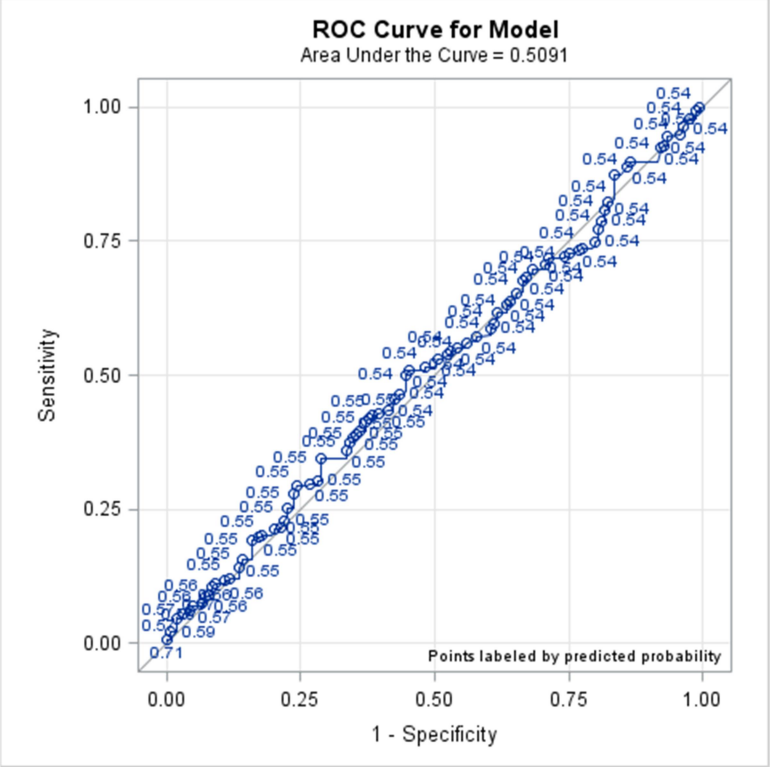
TABLE II – Spearman's Correlations of Exposure (years), Age, KIF5A, KIF18A, and p53 Autoantibodies[a]

	Exposure	Age	KIF5A	KIF18A	p53AAbs
Exposure	1				
	358				
Age	0.3332	1			
	<.0001				
	354	358			
KIF5A	0.3151	-0.1102	1		
	<.0001	0.0371			
	358	358	362		
KIF18A	-0.2157	-0.2885	0.0775	1	
	<.0001	<.0001	0.1412		
	358	358	362	362	
p53AAbs	0.0870	0.3751	-0.1566	-0.1132	1
	0.1003	<.0001	0.0028	0.0313	
	358	358	362	362	362

[a] Each cell presents Spearman's rho, p-value, and number of samples.

The ROC analysis for p53 autoantibody expression confirmed that it was not significant as a predictor of exposure status (p=0.6052) and no cut point could be determined that adequately distinguished exposed from unexposed individuals (Figure 1).

Figure 1. p53 AAbs ROC Curve



Both KIF5A and KIF18A were highly significant predictors of exposure status (p<0.0001) that performed reasonably well at distinguishing exposed from unexposed individuals as shown in

Figures 2 and 3 and allowed determination of optimal cut points to distinguish exposed from unexposed individuals.

Figure 2. KIF5A ROC Curve

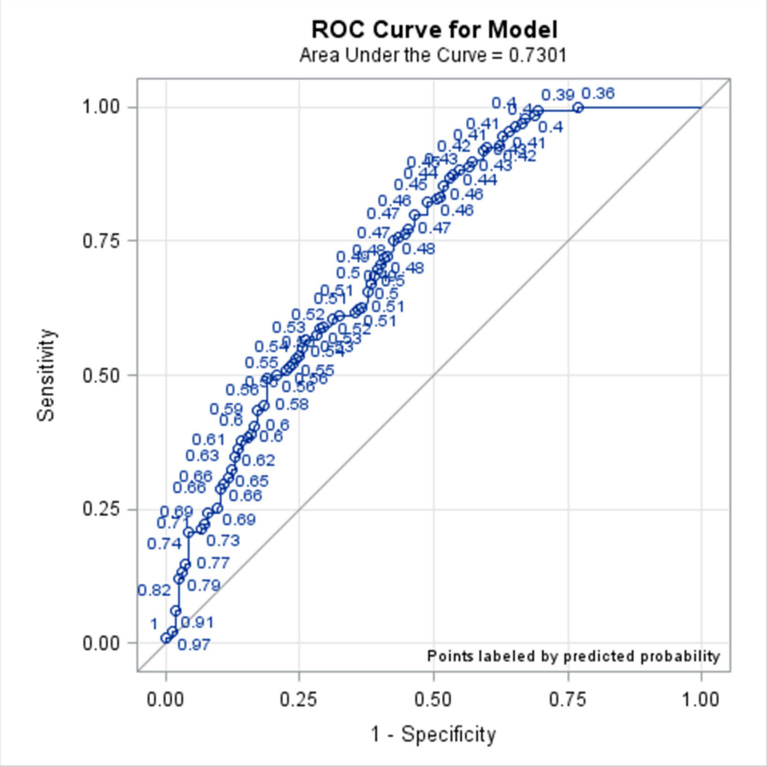
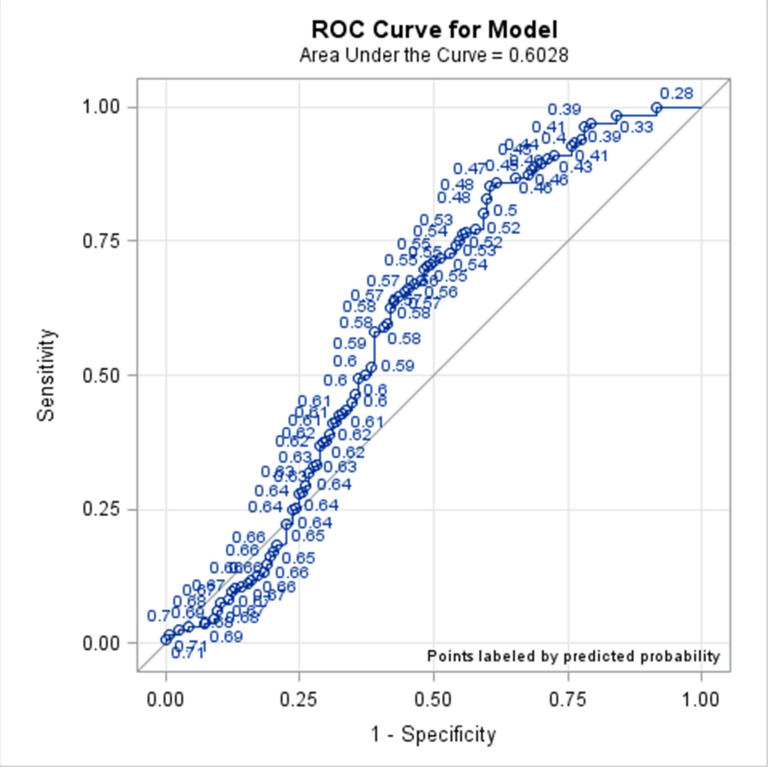


Figure 3. KIF18A ROC Curve



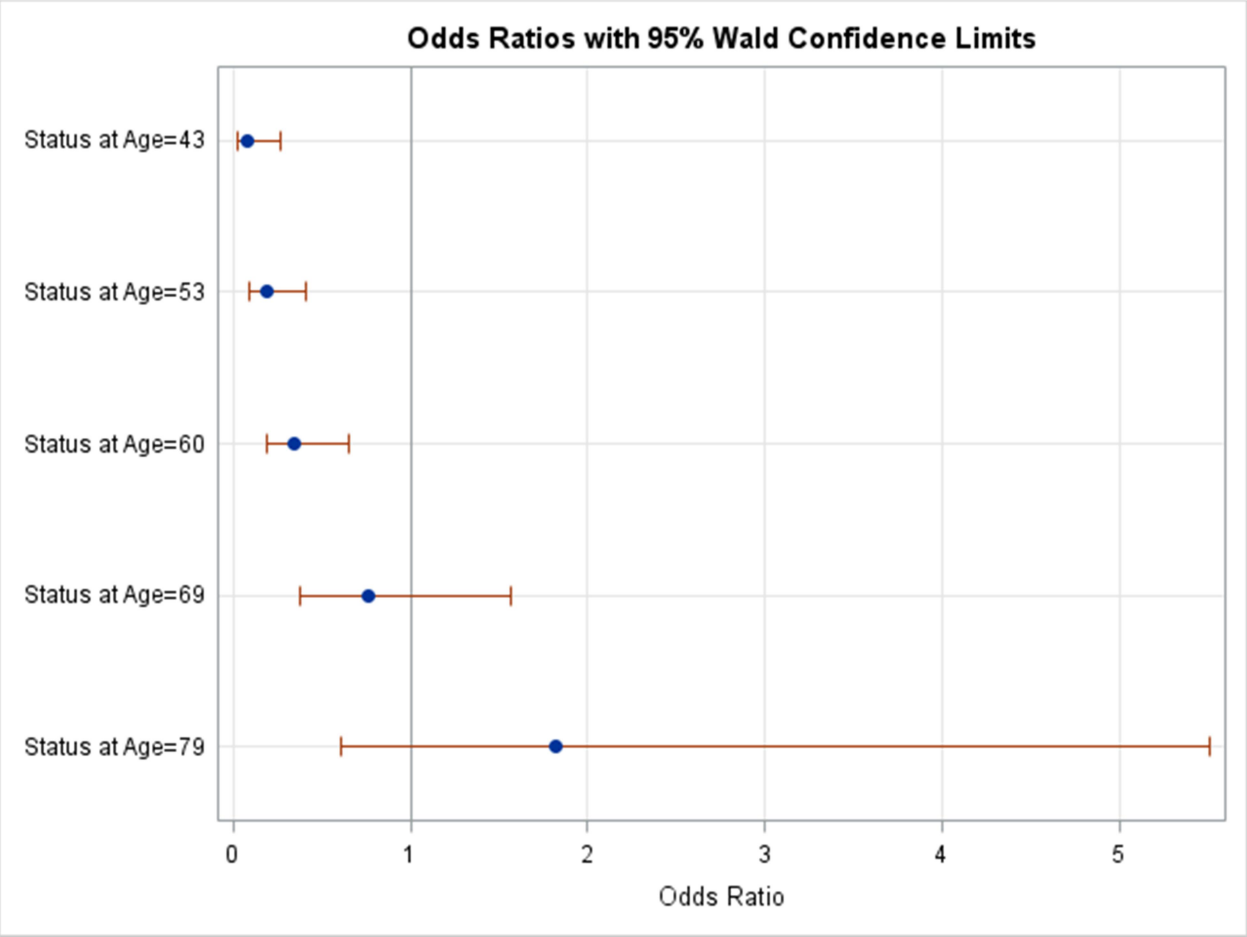
Based upon each cut point determined for the kinesin biomarkers from ROC analyses, individuals were classified as positive or negative for KIF5A and KIF18A, which then served as the outcome in multivariable logistic regression models. The optimal cut point using the Youden's statistic method corresponded to 0.681 ng/mL for KIF5A and 526.399 ng/mL for KIF18A. The optimal cut point using the Euclidean distance method corresponded to 0.901 ng/mL for KIF5A and 361.436 ng/mL for KIF18A, demonstrating heterogeneity among methods of selecting the optimal cut point. Due to this heterogeneity both cut points were used in

assessing the associations of these binary biomarkers to asbestos exposure in multivariable models.

In multivariable models assessing KIF5A positive status as defined by the Youden's statistic cut point, only exposure status (positive/negative) and age were statistically significant, both at $p<0.0001$. Exposed individuals had 7.02 times the odds of positive KIF5A status compared to controls, while each additional year of age had 0.954 times the odds of positive KIF5A status (Table III). In a model using continuous exposure instead of binary exposure status one additional year of exposure had 1.07 times the odds of positive KIF5A status. In multivariable models assessing KIF18A positive status as defined by the Youden's statistic cut point, exposure status (positive/negative) ($p=0.0003$) and age ($p<0.0001$) and the interaction term for status and age ($p=0.0016$) were statistically significant. Exposed individuals had significantly lower odds of positive KIF18A status compared to controls at the 10^{th}, 25^{th}, and 50^{th} percentiles of the age distribution, while exposure status wasn't significantly associated with KIF18a status at the 75^{th} and 90^{th} percentiles of age (Figure 4). Results using continuous exposure were similar with significantly lower odds of positive KIF18A status for each year of asbestos exposure at the 10^{th}, 25^{th}, and 50^{th} percentiles of the age distribution and a non-significant association at the 75^{th} and 90^{th} percentiles of age. Multivariable modeling results were similar using biomarker status for KIF5A and KIF18A defined by the Euclidean distance method. Model fit was better for the Youden's statistic method compared to the Euclidean distance method as measured by area under the curve for KIF5A ($c=0.767$ vs $c=0.704$) and about equal for KIF18A ($c=0.747$ vs $c=0.746$). For this reason the Youden's statistic defined models were selected as the final models. In multivariable models assessing p53 autoantibody

status (positive/negative) as defined by the assay protocol, neither exposure (p=0.89), ex-smoker status (p=0.30), current smoker (p=0.41) status, or age (p=0.13) were significant.

Figure 4. Odds Ratios of Positive KIF18A Status for Asbestos Exposure at the 10th, 25th, 50th, 75th, and 90th Age Percentiles[a]

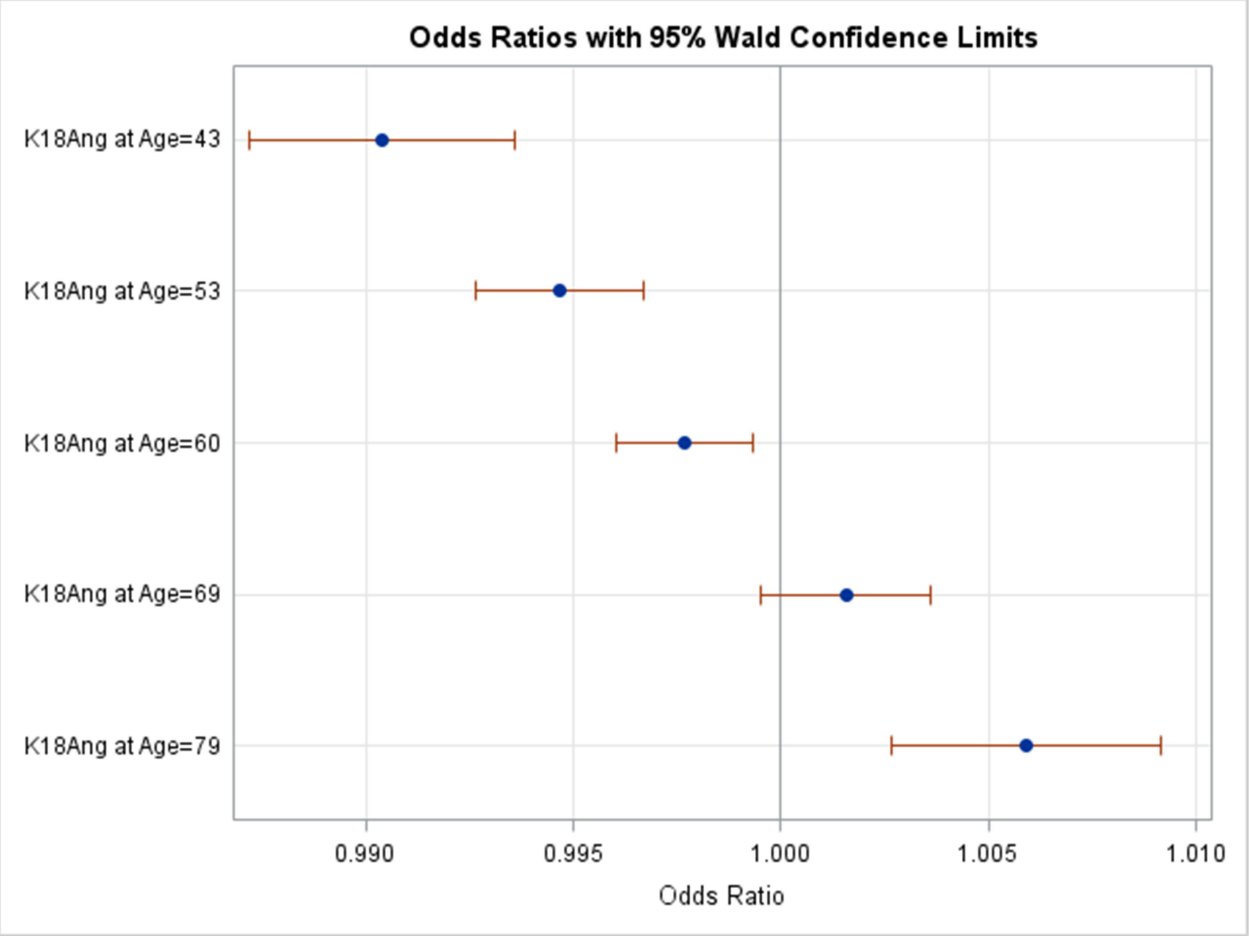


[a] 'Status' is asbestos exposure status. Odds Ratios presented are for positive KIF18A biomarker status (defined by the Youden statistic method) comparing asbestos exposed to unexposed status.

In multivariable logistic regression models of exposure status (positive/negative) including all three biomarkers (continuous) as well as the covariates of age and smoking only KIF5A, KIF18A, age, and the interaction term for KIF18A and age were significant (all $p<0.0001$) (Table III). Individuals with 1 ng/mL higher KIF5A serum levels had 1.87 times the odds of asbestos exposure, while individuals with higher KIF18A serum levels had lower odds of asbestos exposure at younger ages, non-significantly increased odds of exposure at age 69, and significantly increased odds of exposure at age 79 (Figure 5). These findings were generally consistent with results from models examining KIF5A or KIF18A alone and with the associations from models of each biomarker as the outcome of interest.

Similar final models and significance levels were obtained utilizing dichotomous biomarkers as defined by either Youden's statistic or the Euclidian distance method, however the Youden's defined model achieved a better model fit by area under the curve comparison, ($c=0.795$) versus the Euclidean method ($c=0.76$) and therefore was selected as the final model method. Individuals who were positive for KIF5A had 9.56 times the odds of asbestos exposure, while those who were positive for KIF18A had 0.29 times the odds of asbestos exposure compared to those who were negative for each biomarker, and for each additional year of age individuals had 1.05 times the odds of asbestos exposure (Table III). As a predictor, KIF5A positive status had a sensitivity of 85.35%, specificity of 47.56%, positive predictive value of 66.27%, and negative predictive value of 72.9% for asbestos exposure. As a predictor, KIF18A negative status had a sensitivity of 85.35%, specificity of 39.63%, positive predictive value of 69.15%, and negative predictive value of 63.06%.

Figure 5. Odds Ratios of Asbestos Exposure for KIF18A Serum Concentrations at the 10th, 25th, 50th, 75th, and 90th Age Percentiles[a]



[a] Odds Ratio is for a 1 ng/mL increase in serum KIF18A concentration.

TABLE III - Final Regression Model Results for Associations of KIF5A, KIF18A, and Asbestos Exposure[a]

	KIF5A			KIF18A			Age			Current Smoker			Former Smoker		
	beta-coefficient	p-value	OR (95% CI)	beta-coefficient	p-value	OR (95% CI)	beta-coefficient	p-value	OR (95% CI)	beta-coefficient	p-value	OR (95% CI)	beta-coefficient	p-value	OR (95% CI)
Outcome: Exposure															
Model 1	0.5978	<.0001	1.82 (1.43, 2.32)				0.0376	<.0001	1.04 (1.02, 1.06)	-0.2692	0.405	0.76 (0.41, 1.44)	0.2134	0.4431	1.24 (0.72, 2.14)
Model 2				-0.0265	<.0001	[b]	-0.1329	<.0001	[b]	-0.4656	0.1697	0.63 (0.32, 1.22)	0.1601	0.5802	1.17 (0.67, 2.07)
Model 3	0.6234	<.0001	1.87 (1.45, 2.39)	-0.0283	<.0001	[b]	-0.131	<.0001	[b]	-0.4327	0.2249	0.65 (0.32, 1.31)	0.1623	0.5956	1.18 (0.65, 2.14)
Model 4	2.2571	<.0001	9.56 (5.12, 17.8)	-1.242	<.0001	0.29 (0.16, 0.53)	0.0458	<.0001	1.05 (1.03, 1.07)	-0.2266	0.5085	0.8 (0.41, 1.56)	0.303	0.3121	1.35 (0.75, 2.44)

	Exposed Status			Age			Current Smoker			Former Smoker		
	beta-coefficient	p-value	OR (95% CI)	beta-coefficient	p-value	OR (95% CI)	beta-coefficient	p-value	OR (95% CI)	beta-coefficient	p-value	OR (95% CI)
Outcome: KIF5A	1.9488	<.0001	7.02 (3.98, 12.37)	-0.0469	<.0001	0.95 (0.94, 0.97)	0.0019	0.9959	1.0 (0.48, 2.1)	-0.3136	0.3279	0.73 (0.39, 1.37)
Outcome: KIF18A	-6.2869	0.0003	[c]	0.0525	<.0001	[c]	0.2484	0.4886	1.28 (0.64, 2.59)	-0.2449	0.4627	0.78 (0.41, 1.51)

[a] Models 1-3 use continuous biomarkers; Model 4 uses dichotomous biomarkers defined by Youden's Index. All Models (n=335) as 27 individuals with missing data were dropped.

[b] KIF18A and Age had a significant interaction and therefore simple OR is not presented, Figure 5 presents ORs for KIF18A by various ages.

[c] Exposure and Age had a significant interaction (p=0.0016) and therefore simple OR is not presented, Figure 4 presents ORs for Asbestos Exposure by various ages.

4.4 Discussion

Previous studies highlighted in a 2010 review paper have shown that kinesin proteins are overexpressed in several types of cancers and found to be involved in tumorigenesis and metastasis of breast cancer, renal cell carcinoma, nervous system tumors, lung tumors, retinoblastoma tumors, and cervical cancer (Yu and Feng, 2010). It has been shown that KIF18A is essential in the congression of chromosomes and the accurate alignment of the spindle equator and to suppress kinetochore movements during mitosis (Stumpff et al., 2008). Additionally, KIF18A overexpression has been positively related to tumor size and clinical tumor-node-metastasis in hepatocellular carcinoma (HCC), tumor grade and metastasis in breast cancer, and tumor stage, lymphatic invasion, lymph node metastasis, venous invasion, and peritoneal dissemination in colorectal cancer (CRC) as well as poor clinical outcomes (Shichijo et al., 2005; Liao et al., 2014; Zhang et al., 2010; Nagahara et al., 2011).

In our present study we show that KIF18A serum concentrations are significantly decreased in asbestos-exposed individuals compared to unexposed controls, but that the relationship between KIF18A and asbestos exposure varies with age. The inverse association between KIF18A serum levels and asbestos exposure is greatest at lower ages in this cohort (40 to 60 years of age), becomes non-significant by later ages (69 years), and at 79 years of age becomes a statistically significant direct association between increased KIF18A serum levels and asbestos exposure. Lung is one of the few normal tissue types where KIF18A is detectable and this decrease in its serum concentration in younger and middle aged asbestos-exposed individuals may signal an early disruption of its expression and function in the lung tissue. Interestingly the

association was strongest in younger aged individuals and then reversed to a significant increase in serum KIF18A concentrations in older individuals, which may indicate differences in physiologic responses to potential respiratory insult/damage or an already present adaptive response in older individuals, perhaps including higher turnover of cells or increased activity of cells.

While less is known about the role of KIF5A and its potential relationship to disease, our present study has shown significantly increased serum concentrations of KIF5A in asbestos-exposed individuals compared to unexposed controls. This increase in serum KIF5A concentrations may signal an increase in cell activity and/or cell turnover as an early response to asbestos exposure. Both of these changes in KIF18A and KIF5A serum concentrations may be related to early changes that precede development of disease as increased KIF5A concentrations and decreased KIF18A concentrations were found to distinguish asbestos cancer patients from non-cancer controls in a subset of occupationally exposed Finnish asbestosis patients (Tooker et al., 2011).

We additionally found that serum p53 autoantibody concentration was not related to asbestos exposure in this cohort of Italian workers. This finding is in contrast to the borderline significant association between p53 autoantibodies and cumulative asbestos found in a cohort of Finnish asbestosis patients (Li et al., 2005). Additionally, p53 autoantibodies were not significantly associated with smoking status, which was also found in the cohort of Finnish asbestosis patients (Li et al., 2005). Given the well-established relationship between p53 mutations and p53 autoantibodies and numerous cancers including asbestos-related cancers,

our finding of an absence of a relationship to asbestos exposure may indicate that p53 mutations and subsequent autoantibody formation occur nearer to the development of malignant changes and overt disease.

Strengths of this study include the investigation of two new potential biomarkers of asbestos exposure which may reflect a potentially early effect in the disease process as well as an examination of the potential relationship between p53 autoantibodies and asbestos exposure in a new cohort including occupationally exposed workers with a relatively long average cumulative exposure to asbestos. We were additionally able to examine the potential impact of smoking upon these relationships. Our analyses found consistent magnitude and strength of associations for KIF5A, a potential inverse association of KIF18A in younger and middle ages and a potential direct association of KIF18A in the oldest individuals, and lack of association for p53 autoantibodies with asbestos exposure utilizing different approaches. This research does however have several limitations including highly variable data on smoking that necessitated use of current/ex/never classifications, limited data on actual asbestos exposures consisting only of years of presumed exposure, and only one blood measurement used to quantify representative biomarker levels for each individual. The two kinesin ELISAs had high variability in the serum concentrations found among our study population and had much larger inter-assay coefficients of variation than were reported by the manufacturer. The p53 ELISA intra-assay CV was acceptable, but also had a higher than desirable inter-assay CV. While our inter-assay CVs were calculated from a small number of replicates and would have benefitted from more replicates having been run on each plate, this may reflect some inaccuracy in the ELISA kits or procedure that may have biased our findings either toward finding a relationship

that does not exist or failing to find a relationship that does exist between the serum kinesin concentrations and asbestos exposure. Additionally, while the control group did contain a number of other blue collar and white collar workers it was largely composed of healthcare workers who may have differed from asbestos workers on other unmeasured confounders such as diet, exercise, and other lifestyle factors, and exposure to other occupational or environmental pollutants with health risks.

The results from this study suggest that KIF5A, and possibly KIF18A, could serve as useful biomarkers of asbestos exposure and may signal early changes in cellular functioning that could precede the development of asbestos related disease. While the findings for KIF5A were consistent, the potentially varying association observed for KIF18A and asbestos exposure by age may limit its utility as a reliable biomarker. More research is needed to clarify the potential relationships of these proteins to asbestos exposure to determine if the associations found in this population of occupationally exposed individuals are consistent in other occupationally exposed populations as well as possibly in individuals who would only be environmentally exposed to asbestos. Additional studies could also help to clarify the optimal cut point of each kinesin for the molecule to serve as a useful tool for screening. Studies should also be conducted to assess a potential relationship of these molecules to the development of asbestos related disease. This would help to broaden our understanding of the potential role of these kinesins in response to asbestos exposure as potential markers of early effect.

5. SERUM KIF5A AND KIF18A CONCENTRATIONS AS POTENTIAL BIOMARKERS OF ASBESTOS-RELATED CANCER RISK

5.1 Background

The WHO estimates that 125 million people are occupationally exposed to asbestos worldwide and OSHA has estimated that as many as 1.3 million workers in construction and general industry face significant exposures to asbestos on the job in the U.S. (WHO, 2014; IARC 2012). Asbestos exposure is known to result in a number of negative health effects, many of which have long latency periods of up to 20-40 years, including cancer and there has been no evidence of a threshold for the carcinogenic effect of asbestos (ATSDR, 2001; IARC, 2012; WHO 2006). The WHO has also raised its estimates of the global burden of asbestos related disease to 107,000 annual deaths primarily from asbestos related lung cancer, mesothelioma, and asbestosis (WHO, 2014). Given the large population occupationally exposed to asbestos, potential biomarkers of exposure and/or of early effect for cancer risk from asbestos could have a large effect on the burden of disease by identifying individuals at the highest risk for malignancy who could be targeted for more aggressive intervention.

Previous studies using banked serum samples from a cohort of Finnish asbestosis cases who were followed up for subsequent development of cancer have characterized a number of potential biomarkers in their ability to identify individuals at increased risk for occurrence of cancers resulting from asbestos exposure (Brandt-Rauf et al., 1992; Partanen et al., 1994a, 1994b, 1995; Hemminki et al., 1996; Husgafvel-Pursiainen et al., 1997; Li et al., 2005). While combinations of these biomarkers have resulted in relatively high positive predictive value

(0.76) and specificity (0.85), the sensitivity has remained less than desirable (0.51) and so additional work was done to discover new biomarkers with increased sensitivity (Tooker et al., 2011).

Recent examination of a subset of the stored serum samples was undertaken using a proteomic approach based on surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry, an approach which has shown promise for the discovery of new biomarkers of cancer risk (Tooker et al., 2011; Diamandis, 2004). The approach was applied to single serum samples for 35 asbestos workers with asbestosis who subsequently developed cancers (4 malignant mesotheliomas and 31 lung cancers) and 35 asbestos workers with asbestosis who did not develop cancer, group matched for age, gender, race, and smoking status. The sample analyzed for each worker was that closest to, but prior to, the diagnosis of cancer for the 35 cases and the most recent sample for the 35 non-cancer controls. The average time interval between sample collection and the subsequent diagnosis of cancer was 3.7 years (range=1-14 years). The results identified three protein peaks that could predict the development of cancer with good sensitivity (0.87) and specificity (0.70). While one minor peak did not correspond to any known protein, the other two protein peaks were identifiable by protein isolation, digestion, and sequencing analyses and correspond to two members of the kinesin superfamily of proteins, KIF5A and KIF18A (Tooker et al., 2011). The active movement of KIFs supports several critical cellular functions such as mitosis, meiosis, and the transport of macromolecules, for example, through axonal transport. Different subtypes of KIFs may participate in different cellular functions, but KIF5s and KIF18s primarily participate in mitosis and have been suspected to be involved in the carcinogenic process (Yu and Feng, 2010).

This study examines the potential relationship of alterations in KIF5A and KIF18A expression to see if they may be potential biomarkers of increased risk of the development of asbestos-related cancers.

5.2 Materials and Methods

5.2.1 Cohort

In 1978-79 a cohort of 259 pneumoconiosis patients was assembled at the Finnish Institute of Occupational Health in Helsinki (Brandt-Rauf et al., 1992). These were all Finnish workers with compensable asbestosis or silicosis who were referred to the Institute for further evaluation and who fulfilled the usual diagnostic criteria for their disease. The cohort included 115 cases of asbestosis and 144 cases of silicosis who were planned to be followed prospectively for at least ten years to evaluate the course of their disease. The baseline evaluations in 1978-79 included complete medical histories, including demographic data and occupational and smoking histories, physical examinations, spirometry and chest radiographs. On annual visits between March 1980 and August 1987 blood samples were also requested of some of the participants (particularly the asbestosis cases), and for those participants who consented, blood samples were collected by routine venipuncture techniques and 2 ml aliquots of serum were separated and stored frozen at -70^{o}C. For various reasons, some cases were lost to follow-up, failed to show up for every scheduled appointment or refused to give blood samples. Excluding these, the cohort consists of those 110 cases of asbestosis with at least one available stored serum samples and follow-up as to health status through the end of 2007. The cohort is thus composed of 110 Finnish workers with asbestosis, 102 of whom are male (93%)

and 8 of whom are female (7%). The average age of the subjects at the end of sample collection in 1988 was 66.8 years with a range of 40-89 years (2 in their 40s; 22 in their 50s; 42 in their 60s; 33 in their 70s; 11 in their 80s). Most cases had many years of estimated exposure to asbestos (average=20 years; range=2-44 years) in job categories with high likelihood of asbestos exposure (asbestos insulator - 30%; asbestos miner - 24%; asbestos cement worker - 19%; asbestos sprayer - 10%; other miscellaneous asbestos worker - 17%). As a result, estimated exposures were relatively high with an average estimated cumulative exposure of 523 fiber-years/mL (range=14-1750 fiber-years/mL). The cohort includes 27% non-smokers, 44% ex-smokers, and 29% current smokers (58% of whom average 1-14 cigarettes/day, 38% of whom average 15-24 cigarettes/day, and 4% of whom average more than 24 cigarettes/day). It should be noted that the vast majority of subjects in this study are white males. This is due to the fact that in the asbestos industry in Finland until very recently, these jobs were almost exclusively occupied by white males.

Cancer incidence within the cohort was followed up through December 31, 2007 from the Finnish Cancer Registry, a national registry with a complete coverage of diagnosed cancers in the country. At that time, 55 of the 110 asbestosis cases had developed malignant tumors including 34 lung cancers (10 squamous cell carcinomas, 5 adenocarcinomas, 6 small cell carcinomas, 2 bronchoalveolar carcinomas, 7 anaplastic carcinomas, and 4 lung cancers that were not further specified as to type), 4 malignant pleural mesotheliomas, and 17 other cancer of various types (larynx, colorectum, esophageal, urinary bladder, kidney, pancreas, prostate, brain, gallbladder, melanoma and lymphoma). The remaining 55 asbestosis cases in the cohort were followed up through the Finnish Institute of Occupational Health and confirmed to have

not developed any malignant tumor as of the end of 2007 and either be still living (13) or having died of other causes (42).

As noted, all 110 patients in this cohort had at least one serum sample collected between 1980 and 1987 available for ELISA analysis. All serum samples have been kept frozen at -70°C since the time of collection and have been randomly recoded so that the analyses were performed blinded to subject identity and case/control status. For the 55 asbestosis cases without cancer there are 196 serum samples available ranging from 1-7 per subject (10 subjects with 1 sample, 9 subjects with 2 samples, 6 subjects with 3 samples, 13 subjects with 4 samples, 8 subjects with 5 samples, 5 subjects with 6 samples, and 4 subjects with 7 samples), and for the 55 asbestosis cases with cancer there are 168 serum samples available ranging from 1-7 per subject (15 subjects with 1 sample, 9 subjects with 2 samples, 8 subjects with 3 samples, 11 subjects with 4 samples, 6 subjects with 5 samples, 5 subjects with 6 samples, and 1 subject with 7 samples), all collected prior to the date of cancer diagnosis which ranges from 1981 to 2007.

5.2.2 Laboratory Procedures

Serum samples were thawed and analyzed for the presence of KIF5A and KIF18A proteins at the University of Illinois at Chicago by commercially available ELISAs in 2013. In both cases, the assays are quantitative sandwich ELISAs utilizing microtiter plates pre-coated with a monoclonal antibody specific for the particular KIF. After incubation of the sample, a biotin-conjugated polyclonal antibody specific for the particular KIF is added followed by avidin-conjugated horseradish peroxidase and 3,3',5,5'-tetramethylbenzidine substrate solution. The color

change is measured spectrophotometrically at 450 nm and is converted into the concentration of KIF in the sample by comparison to a standard curve generated from known concentrations of purified KIF protein (run in duplicate) on each plate. The KIF concentration of each sample/individual was then compared to a cutoff level to determine positive or negative status for altered serum protein concentrations. The optimal cutoff level for KIF5A and KIF18A protein concentrations was empirically determined utilizing ROC analyses. These ELISA assays have been demonstrated to be highly reproducible and to have high sensitivity (LLD<118 pg/mL) and specificity (no cross-reactivity between each specific KIF and known analogues). The manufacturer reported intra-assay coefficient of variation is <10% and inter-assay coefficient of variation is <12%. We were unable to calculate intra-assay CV, but our calculated inter-assay CV was 31.74%. Finally, from the ELISA results comparisons can be made between the KIF levels found in serum and the subsequent development of cancer, as described below; this analysis was applied to all cancers in the cohort as well to cancers most likely related to asbestos exposure, such as lung cancers, mesotheliomas, esophageal cancer, and colorectal cancer.

5.2.3 Data Analysis

Distributions of KIF5A and KIF18A (continuous) were evaluated for normality and Spearman correlations were assessed between the continuous biomarkers, continuous exposure (years and fiber-years) and age. Individuals with missing variables were dropped from the analysis (n=1 missing age; n=1 missing gender; n=1 missing smoking status; n=1 missing exposure years and fiber-years; n=1 total missing (1.3%)). Mean levels of each potential biomarker were compared in relation to cancer diagnosis via parametric and non-parametric analyses where appropriate as well as via receiver operating characteristic (ROC) analysis. Receiver operating

characteristic analysis is a method of analyzing the predictive or discriminatory performance of a potential biomarker or diagnostic test by plotting the sensitivity (true positive rate) against 1-specificity (the false positive rate) for a range of potential cut-points. From this plot one can determine the optimal cut point for each biomarker to differentiate between a binary outcome, in this case cancer diagnosis, utilizing various methods including Youden's statistic and Euclidian distance from the perfect classifier (point 0, 1) (Youden, 1950). Generalized estimating equations were used for the logistic regression models for the ROC analysis to account for multiple samples per individual. Univariate logistic regression models for each biomarker on other cancers as well as for cancers most directly related to asbestos exposure, such as lung cancers and mesotheliomas were run for the ROC analysis. Additional multivariate logistic regression models were run for each biomarker, as well as the two biomarkers combined, and each cancer outcome to examine potential relationships in the context of age, smoking history, and asbestos exposure.

5.3 Results

General descriptive statistics on the cohort including biomarker expression levels and the measured covariates of age and smoking status are presented in Table IV. Asbestos-related cancer cases were significantly younger than control patients ($p<0.0001$). There was no statistically significant difference in asbestos exposure years ($p=0.2927$), but fiber-years ($p=0.0361$) were significantly higher in asbestos-related cancer cases compared to non-cancer cases. Smoking status was significantly different among cases and controls ($p<0.0001$), with higher percentages of cases being former smokers and of controls being current and never

smokers. There was no statistically significant difference in gender between asbestos-related cancer cases and non-cases (p=0.1070).

TABLE IV - Serum KIF5A, KIF18A, Asbestos Exposure, Age, Gender, and Smoking Status of Asbestosis Patients by Asbestos-Related Cancer Status[a]

	Asbestos Related Cancer Cases (n=28, 35.4%)	Individuals Without Asbestos Related Cancers (n=51, 64.6%)	[b]
Age at Sample Collection (years)	56.1 (6.9)	59.1 (8.8)	(p<0.0001)*
Gender			(p=0.1070)
Male	26 (92.9%)	45 (88.2%)	
Female	1 (3.6%)	6 (11.8%)	
Smoking			(p<0.0001)*
Current	9 (32.1%)	26 (51%)	
Former	17 (60.7%)	11 (21.6%)	
Never	1 (3.6%)	14 (27.5%)	
Asbestos exposure (years)	21.5 (8.3)	20.3 (9.2)	(p=0.2927)
Asbestos exposure (fiber-years)	561.3 (438.4)	479.4 (509.4)	(p=0.0361)*
KIF5A (ng/mL)	3.91 (2.8)	3.3 (2.29)	(p=0.9256)
KIF18A (ng/mL)	434.3 (166.9)	425.4 (167.4)	(p=0.2305)

[a] Age, asbestos exposure (years and fiber-years), KIF5A, and KIF18A are represented as: mean (standard deviation); Gender and smoking are represented as: n (%).

[b] p-values from Wilcoxon rank-sum test for Age, Asbestos exposure (years and fiber-years), KIF5A, KIF18A, and Chi squared test for gender and smoking. * indicates significant p<0.05

Both KIF5A and KIF18A were found to be log-normally distributed by statistical tests for normality including the Shapiro-Wilk (p<0.0001), Kolmogorov-Smirnov (p<0.01) and Anderson-Darling (p<0.005). Therefore potential differences in serum biomarker levels between cancer

cases and control patients were assessed on log-transformed biomarker values or via the non-parametric Wilcoxon rank-sum test. There were no statistically significant differences between serum levels of KIF5A or KIF18A between various groups of cancer patients including asbestos-related cancer patients (Table IV), lung and mesothelioma patients combined, lung cancer patients, mesothelioma patients, other cancer patients, and all cancer patients when compared to non-cancer control patients.

TABLE V – Spearman Correlations of Exposure (years and fiber-years), Age, KIF5A, and KIF18A[a]

	KIF5A	KIF18A	Age	Exposure (years)	Exposure (fiber-years)
KIF5A	1				
	244				
KIF18A	-0.0020	1			
	0.9753				
	244	244			
Age	0.0318	-0.1572	1		
	0.6233	0.0146			
	241	241	241		
Exposure (years)	-0.1077	0.0778	0.3189	1	
	0.0954	0.2286	<.0001		
	241	241	241	241	
Exposure (fiber-years)	-0.0561	0.1269	-0.2871	0.4245	1
	0.3856	0.0491	<.0001	<.0001	
	241	241	241	241	241

[a] Each cell presents Spearman's rho, p-value, and number of samples

Correlations were assessed between continuous variables to explore potential associations and found that KIF18A was inversely correlated with age (rho= -0.16, p=0.0146) and borderline significantly with exposure (fiber-years) (rho=0.13, p=0.0491); age was directly correlated with exposure years (rho=0.32, p<0.0001), but was inversely associated with fiber-years (rho= -0.29, p<0.0001); and exposure years were significantly correlated with fiber-years (rho=0.42, p<0.0001) (Table V). Neither KIF5A nor KIF18A were significantly correlated with exposure (years) and KIF15A was not significantly correlated with fiber-years. Additionally, KIF5A levels were significantly lower (p<0.05) in never smokers compared to current smokers or former smokers, but there were no significant differences in KIF18A levels between current, former, or never smokers. There were no significant differences in KIF5A or KIF18A levels between males and females.

Separate ROC models were run to assess the predictive performance and optimal cut-point of KIF5A and KIF18A for asbestos-related cancer, lung cancer and mesothelioma, lung cancer, other cancers, and all cancers as the outcome in logistic regression models utilizing the generalized estimating equations method to account for repeated measures for individuals (Table VI). Neither biomarker was significant in any model and the models performed no better than chance at discriminating cases from non-cases as demonstrated by the p-values and c-statistic values shown in Table VI. The ROC curves of asbestos-related cancers are presented for KIF5A (Figure 6) and KIF18A (Figure 7).

TABLE VI – Summary of ROC Model Results for Kinesins as Predictors of Several Cancers[a]

Model	KIF5A		KIF18A	
	p-value	c statistic	p-value	c statistic
Asbestos Related	0.6019	0.504	0.3844	0.548
Lung & Mesothelioma	0.1823	0.535	0.4671	0.541
Lung	0.1691	0.548	0.1707	0.57
Other	0.5191	0.513	0.2027	0.579
All	0.4843	0.521	0.7044	0.519

[a] P-value indicates statistical significance of the kinesin as a predictor of the cancer outcome and c statistic represents the area under the curve for each ROC curve.

Figure 6. KIF5A ROC Curve for Asbestos-Related Cancers

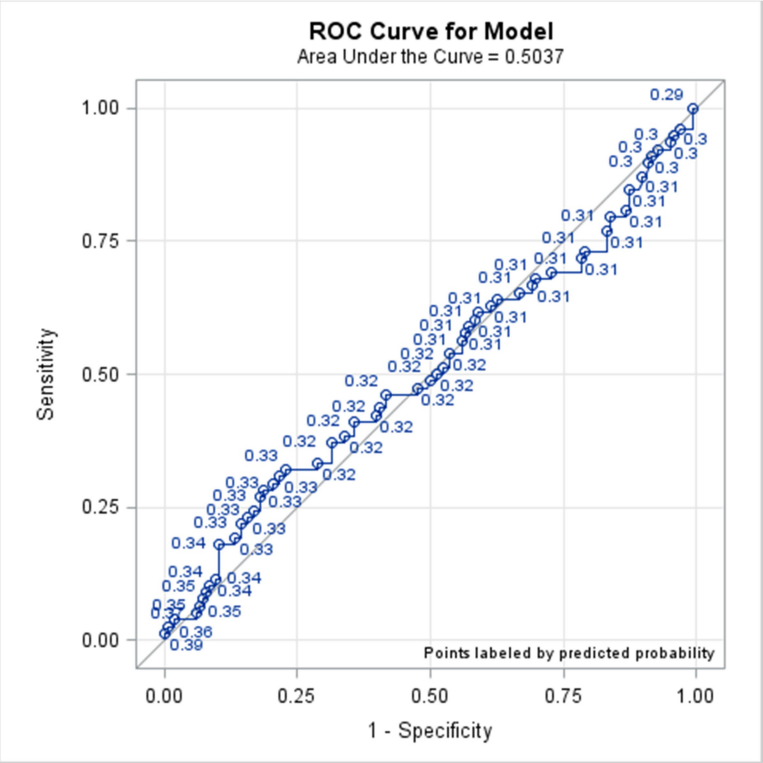
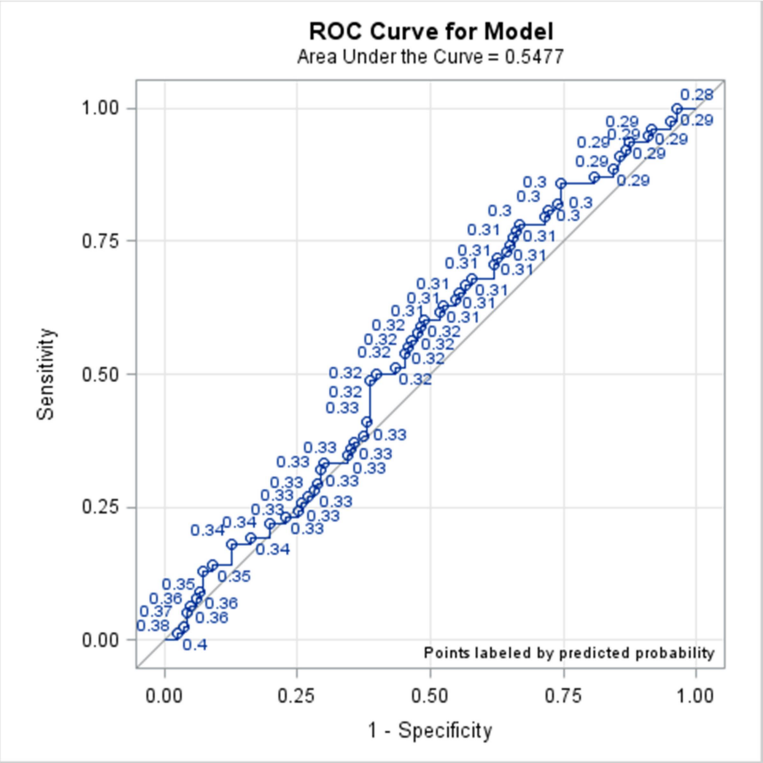


Figure 7. KIF18A ROC Curve for Asbestos-Related Cancers



Additional generalized estimating equation logistic regression models were run using the continuous biomarkers and including the co-variates of age, sex, current and former smoking status, exposure (years), and exposure (fiber-years), and interaction terms for each kinesin and the co-variates for asbestos-related cancers, lung cancer and mesotheliomas, lung cancers, mesotheliomas, other cancers, and all cancers. Neither biomarker was significant in any model as a predictor of cancer outcomes in this population alone or in combination, with final model results shown for each endpoint in Table VII.

TABLE VII - Final Regression Model Results for Associations of KIF5A, KIF18A, and Cancer Diagnoses[a]

	KIF5A			KIF18A			Age			Current Smoker			Former Smoker		
	beta-coefficient	p-value	OR (95% CI)	beta-coefficient	p-value	OR (95% CI)	beta-coefficient	p-value	OR (95% CI)	beta-coefficient	p-value	OR (95% CI)	beta-coefficient	p-value	OR (95% CI)
Model 1 - Asbestos Related	0	0.8993	1.00 (1.0, 1.0)				0	0.5489	1.00 (0.9999, 1.0001)	1.5781	0.1533	4.85 (0.56, 42.26)	3.0742	0.0054	21.6 (2.48, 188.7)
Model 2 - Asbestos Related				0	0.5665	1.00 (1.0, 1.0)	0	0.5109	1.00 (0.9999, 1.0001)	1.5781	0.1533	4.85 (0.56, 42.26)	3.0742	0.0054	21.6 (2.48, 188.7)
Model 3 - Asbestos Related	0	0.8692	1.00 (1.0, 1.0)	0	0.5363	1.00 (1.0, 1.0)	0	0.531	1.00 (0.9999, 1.0001)	1.5781	0.1533	4.85 (0.56, 42.26)	3.0742	0.0054	21.6 (2.48, 188.7)
Model 4 - Other	0	0.6151	1.00 (1.0, 1.0)				0	0.6443	1.00 (0.9999, 1.0001)	-0.0492	0.944	0.95 (0.24, 3.76)	-2.2841	0.0517	0.1 (0.01, 1.02)
Model 7 - All	-0.0012	0.7244	0.9988 (0.99, 1.01)				0.0026	0.7572	1.003 (0.986, 1.019)	1.0715	0.1168	2.92 (0.77, 11.14)	1.6179	0.0235	5.04 (1.24, 20.45)
Model 8 - All				0	0.7479	1.00 (1.0, 1.0)	0.0024	0.7659	1.002 (0.987, 1.018)	1.0688	0.1175	2.91 (0.76, 11.1)	1.6143	0.0238	5.02 (1.24, 20.38)
Model 9 - All	-0.0013	0.719	0.9987 (0.99, 1.01)	0	0.7337	1.00 (1.0, 1.0)	0.0027	0.753	1.00 (0.9858, 1.0199)	1.0715	0.1168	2.92 (0.77, 11.14)	1.6186	0.0235	5.05 (1.24, 20.47)

[a] Odds Ratio (OR) for KIF5A, KIF18A, and Age are for a 1 unit increase (ng/mL) for both kinesins and (years) for age; OR for Current Smoker and Former Smoker are compared to Never Smoker status. Beta-coefficient is the parameter estimate for each variable in the model and p-value indicates statistical significance. All models (n=241) as 3 observations were dropped due to missing values.

5.4 Discussion

Initial results from a subset of this cohort had indicated that KIF5A and KIF18A may be potentially useful biomarkers in distinguishing individuals with asbestosis but no cancer from individuals with asbestosis who also have cancer, by showing increased KIF5A and decreased KIF18A concentrations in the serum of cancer patients compared to non-cancer controls (Tooker et al., 2011), but analysis of the full cohort in the current report did not confirm these initial findings. In the expanded analysis, KIF5A and KIF18A serum levels showed no difference between any of the observed cancer patient populations and their non-cancer controls. This may be due to differences between the subset of cancer patients and controls selected for the initial screening study, as compared to the broader cohort, or due to the limitations of the original method used to measure and identify the potential biomarkers. The size of the Finnish cohort has also been reduced by a third due to sample use in previous studies, which may have impacted our ability to detect statistical differences due to the low sample size as many models failed to converge for several of the cancer subsets. The two kinesin ELISAs had high variability in the serum concentrations found among our study population and had much larger inter-assay coefficients of variation than were reported by the manufacturer. While our inter-assay CVs were calculated from a small number of replicates and would have benefitted from more replicates having been run on each plate, this may reflect some inaccuracy in the ELISA kits or procedure that may have biased our findings either toward finding a relationship that does not exist or failing to find a relationship that does exist between the serum kinesin concentrations and the various cancers. Another potential limitation of our present study was the need to use serum concentrations of the kinesins as a marker, whereas previous studies in the literature

examined differences between expression levels in normal and cancer tissues (Shichijo et al., 2005; Liao et al., 2014; Zhang et al., 2010; Nagahara et al., 2011). Additionally, potential sample degradation could have occurred as these samples were collected several decades before this analysis was conducted and the long-term stability of these kinesin proteins in frozen serum samples is not well characterized. Despite these limitations our study is the first to examine KIF5A and KIF18A in a well characterized cohort of occupationally exposed asbestosis patients with a long follow up for cancer diagnosis.

We have previously shown that KIF5A levels are significantly higher in an Italian cohort of asbestos-exposed individuals free of asbestosis compared to unexposed individuals. Additionally we found that KIF18A serum levels are significantly lower in younger and middle-aged individuals, but significantly higher in older individuals who were exposed to asbestos compared to unexposed individuals. However, in this cohort of Finnish asbestosis patients neither biomarker was significantly associated with asbestos exposure measured in years and only KIF18A was marginally associated with fiber-years of exposure. Perhaps the discrepant results for the exposure-biomarker relationships and the lack of predictive value for cancer risk of either protein in this study reflects some underlying disruption to KIF5A and KIF18A expression that is already present in asbestosis patients who have all had relatively high cumulative asbestos exposure.

Follow up of other occupationally exposed cohorts of asbestos workers and ongoing collection of health data and blood samples for analysis could aid in understanding the kinesins potential role in the exposure-disease pathway. Further research is needed to clarify the

potential relationship of KIF5A and KIF18A to asbestos exposure-related disease and cancer risk, particularly with a larger number of cancer cases in order to increase the statistical ability to detect potential relationships as this study was limited due to low numbers.

6. SERUM KIF5A, KIF18A, AND p53 AUTOANTIBODY CONCENTRATIONS AS POTENTIAL BIOMARERS OF ASBESTOSIS SEVERITY

6.1 Background

The WHO estimates that 125 million people are occupationally exposed to asbestos worldwide and OSHA has estimated that as many as 1.3 million workers in construction and general industry face significant exposures to asbestos on the job in the U.S. (WHO, 2014; IARC 2012). Asbestos exposure is known to result in a number of negative health effects, many of which have long latency periods of up to 20-40 years, including cancer and there has been no evidence of a threshold for the carcinogenic effect of asbestos (ATSDR, 2001; IARC, 2012; WHO 2006). The WHO has also raised its estimates of the global burden of asbestos related disease to 107,000 annual deaths primarily from asbestos related lung cancer, mesothelioma, and asbestosis (WHO, 2014).

Previous studies have identified two members of the kinesin superfamily of proteins, KIF5A and KIF18A, as potential markers of asbestos exposure or asbestos-related cancers (Tooker et al., 2011; Schmitz et al., manuscript in preparation). While the initial finding of a relationship between KIF5A and KIF18A expression and asbestos-related cancer was not confirmed in further analysis of the cohort of Finnish asbestosis patients, the relationship between altered kinesin expression and asbestos exposure in an Italian cohort of asbestos workers suggested that perhaps alterations in kinesin expression lay earlier in the exposure-disease pathway. The fact that KIF5A and KIF18A did not show a significant relationship with asbestos exposure measured in years or fiber-years in the Finnish cohort of asbestosis patients may also suggest

that kinesin perturbation is already present in this cohort where all members had relatively high cumulative exposures to asbestos.

Serum p53 autoantibodies have been found in patients with a number of pre-malignant diseases and cancers and in workers exposed to occupational carcinogens including asbestos before any clinical evidence of malignancy (Cordes et al., 2009; Anderson et al., 2010; Mattioni et al., 2013; Suppiah and Greenman, 2013; Li et al., 2005). The reported sensitivity of p53 autoantibodies as a tumor marker in cancer patients has generally been low, while the specificity as a tumor marker has been high, but because p53 autoantibodies have been found in numerous cancer types, the presence of p53 autoantibodies are not a specific marker for a particular cancer (Cordes et al., 2009; Anderson et al., 2010; Suppiah and Greenman, 2013). Previous investigation of this Finnish cohort found a statistically significant relationship between serum p53 autoantibodies and the subsequent development of malignancy with an average lead time to diagnosis of 3.5 years (Li et al., 2005). That same study showed a borderline statistically significant relationship between p53 autoantibodies and low vs. moderate-high tertiles of cumulative asbestos exposure ($p=0.05$), a relationship that was not found in our study of an Italian cohort of asbestos workers (Li et al., 2005; Schmitz et al., manuscript in preparation).

In an attempt to further clarify the relationship between asbestos exposure, KIF5A, KIF18A, p53 autoantibodies, and asbestos-related disease this study will evaluate whether ILO International Classification of Radiographs of Pneumoconioses scores for the cohort are associated with asbestos exposure, KIF5A, KIF18A and p53 autoantibody serum concentrations.

ILO scores are a clinical indication of the severity of asbestosis and thus an association between the kinesin and/or p53 autoantibody serum concentrations could indicate they could be useful as markers of asbestosis severity and useful as an additional tool to monitor individuals with known asbestos exposure for disease.

6.2 Materials and Methods

6.2.1 Cohort

In 1978-79 a cohort of 259 pneumoconiosis patients was assembled at the Finnish Institute of Occupational Health in Helsinki (Brandt-Rauf et al., 1992). These were all Finnish workers with compensable asbestosis or silicosis who were referred to the Institute for further evaluation and who fulfilled the usual diagnostic criteria for their disease. The cohort included 115 cases of asbestosis and 144 cases of silicosis who were planned to be followed prospectively for at least ten years to evaluate the course of their disease. The baseline evaluations in 1978-79 included complete medical histories, including demographic data and occupational and smoking histories, physical examinations, spirometry and chest radiographs. On annual visits between March 1980 and August 1987 blood samples were also requested of some of the participants (particularly the asbestosis cases), and for those participants who consented, blood samples were collected by routine venipuncture techniques and 2 ml aliquots of serum were separated and stored frozen at -70^{o}C. For various reasons, some cases were lost to follow-up, failed to show up for every scheduled appointment or refused to give blood samples. Excluding these, the cohort consists of those 110 cases of asbestosis with at least one available stored serum samples and follow-up as to health status through the end of 2007. The

cohort is thus composed of 110 Finnish workers with asbestosis, 102 of whom are male (93%) and 8 of whom are female (7%). The average age of the subjects at the end of sample collection in 1988 was 66.8 years with a range of 40-89 years (2 in their 40s; 22 in their 50s; 42 in their 60s; 33 in their 70s; 11 in their 80s). Most cases had many years of estimated exposure to asbestos (average=20 years; range=2-44 years) in job categories with high likelihood of asbestos exposure (asbestos insulator - 30%; asbestos miner - 24%; asbestos cement worker - 19%; asbestos sprayer - 10%; other miscellaneous asbestos worker - 17%). As a result, estimated exposures were relatively high with an average estimated cumulative exposure of 523 fiber-years/mL (range=14-1750 fiber-years/mL). The cohort includes 27% non-smokers, 44% ex-smokers, and 29% current smokers (58% of whom average 1-14 cigarettes/day, 38% of who average 15-24 cigarettes/day, and 4% of whom average more than 24 cigarettes/day). It should be noted that the vast majority of subjects in this study are white males. This is due to the fact that in the asbestos industry in Finland until very recently, these jobs were almost exclusively occupied by white males with very few females or minorities, so few females and no minorities can be included in this study. Since this is a study of workers, children are likewise not included in this study.

For evaluation of asbestosis, posterior–anterior chest radiographs were arranged in random order and classified for degree of disease by the consensus of three readers (two radiologists, one of whom was an ILO B-reader, and an internist/occupational medicine physician) who were blinded to the subjects' identities, using the ILO 1980 classification in effect at the time of the study. The distribution of the subjects by their baseline radiographic subcategory was: one case 0/0, 11 cases 0/1, 25 cases 1/0, 29 cases 1/1, six cases 1/2, three cases 2/1, five cases 2/2, one

case 2/3 and one case 3/3; or aggregated by major radiographic category: 12 cases 0, 60 cases 1, nine cases 2 and one case 3. The distribution of the subjects by their worst radiographic subcategory was: one case 0/0, eight cases 0/1, 19 cases 1/0, 21 cases 1/1, three cases 1/2, nine cases 2/1, 10 cases 2/2, six cases 2/3 and five cases 3/3; or aggregated by major radiographic category: nine cases 0, 43 cases 1, 25 cases 2 and five cases 3. Of the 82 subjects, 31 (38%) had radiographic progression of their disease during the period of follow-up during the study, with an increase of from one to six subcategories of the ILO classification (six cases increased one subcategory, 10 cases increased two subcategories, 10 cases increased three subcategories, two cases increased four subcategories, two cases increased five subcategories, one case increased six subcategories). No subjects were judged to have radiographic regression of their disease over the course of the study.

As noted, all 110 patients in this cohort had at least one serum sample collected between 1980 and 1987 available for ELISA analysis. All serum samples have been kept frozen at -70°C since the time of collection and have been randomly recoded so that the analyses were performed blinded to subject identity and case/control status. For the 55 asbestosis cases without cancer there are 196 serum samples available ranging from 1-7 per subject (10 subjects with 1 sample, 9 subjects with 2 samples, 6 subjects with 3 samples, 13 subjects with 4 samples, 8 subjects with 5 samples, 5 subjects with 6 samples, and 4 subjects with 7 samples), and for the 55 asbestosis cases with cancer there are 168 serum samples available ranging from 1-7 per subject (15 subjects with 1 sample, 9 subjects with 2 samples, 8 subjects with 3 samples, 11 subjects with 4 samples, 6 subjects with 5 samples, 5 subjects with 6 samples, and 1 subject

with 7 samples), all collected prior to the date of cancer diagnosis which ranges from 1981 to 2007.

6.2.2 Laboratory Procedures

Serum samples were thawed and analyzed for the presence of KIF5A and KIF18A proteins at the University of Illinois at Chicago by commercially available ELISAs. In both cases, the assays are quantitative sandwich ELISAs utilizing microtiter plates pre-coated with a monoclonal antibody specific for the particular KIF. After incubation of the sample, a biotin-conjugated polyclonal antibody specific for the particular KIF is added followed by avidin-conjugated horseradish peroxidase and 3,3',5,5'-tetramethylbenzidine substrate solution. The color change is measured spectrophotometrically at 450 nm and is converted into the concentration of KIF in the sample by comparison to a standard curve generated from known concentrations of purified KIF protein (run in duplicate) on each plate. The KIF concentration of each sample/individual was then compared to a cutoff level to determine positive or negative status for altered serum protein concentration. The optimal cutoff level for KIF5A and KIF18A protein concentration was empirically determined utilizing ROC analyses. These assays have been demonstrated to be highly reproducible and to have high sensitivity (LLD<118 pg/mL) and specificity (no cross-reactivity between each specific KIF and known analogues). The manufacturer reported intra-assay coefficient of variation is <10% and inter-assay coefficient of variation is <12%. We were unable to calculate intra-assay CV, but our calculated inter-assay CV was 31.74%. Finally, from the ELISA results comparisons can be made between the KIF levels found in serum and the ILO Classification scores.

6.2.3 Data Analysis

Distributions of KIF5A and KIF18A (continuous) were evaluated for normality and Spearman correlations were assessed between the continuous biomarkers, continuous exposure (years and fiber-years) and age. Individuals with missing variables were dropped from the analysis. Mean levels of each potential biomarker were compared in relation to ILO Classification scores via parametric and non-parametric analyses where appropriate as well as via receiver operating characteristic (ROC) analysis. Receiver operating characteristic analysis is a method of analyzing the predictive or discriminatory performance of a potential biomarker or diagnostic test by plotting the sensitivity (true positive rate) against 1-specificity (the false positive rate) for a range of potential cut-points. From this plot one can determine the optimal cut point for each biomarker to differentiate between a binary outcome, in this case asbestosis patients with ILO Classification scores for major categories of 2 and 3 and patients with major categories of 0 and 1, utilizing various methods including Youden's statistic and Euclidian distance from the perfect classifier (point 0, 1) (Youden, 1950). Generalized estimating equations were used for the logistic regression models for the ROC analysis to account for multiple samples per individual. Univariate logistic regression models for each kinesin biomarker on binary ILO Classification score were run for the ROC analysis. p53 autoantibody status (positive/negative) was also assessed in relation to ILO Classification score via the chi-squared test. Multivariable logistic and multinomial regression models were also run to assess the relationship of each biomarker individually and combined in the context of other potential confounders such as age, smoking status, and asbestos exposure.

6.3 Results

General descriptive statistics on the cohort including biomarker expression levels and the measured covariates of age, gender, smoking status, and exposure are presented in Table VIII. Both KIF5A and KIF18A, as well as age and exposure (years and fiber-years) were found to be log-normally distributed by statistical tests for normality including the Shapiro-Wilk ($p<0.001$), Kolmogorov-Smirnov ($p<0.01$) and Anderson-Darling ($p<0.005$). Therefore potential differences in serum biomarker levels, age, and exposure between ILO major category score groups were assessed on log-transformed biomarker values or via the non-parametric Wilcoxon rank-sum test. There were no statistically significant differences between serum levels of KIF5A and ILO severity score groups ($p=0.1741$), but KIF18A did vary significantly by ILO severity score group ($p=0.0014$), with higher KIF18A serum concentrations in groups 3 and 2 compared to group 1 ($p<0.05$). The chi squared test was borderline significant ($p=0.049$) for p53 autoantibody positive status by ILO severity score groups with higher percentages of p53 autoantibody positive patients in higher ILO severity categories, but the low cell counts may have impacted the validity of the test. The chi squared test for p53 autoantibody positive status by binary ILO status was significant ($p=0.02$). Age was significantly different among ILO severity groups ($p=0.0006$), with lower mean age in ILO group 0 compared to ILO group 1 ($p<0.05$). Exposure (years) ($p=0.0270$) and exposure (fiber-years) ($p<0.0001$) were significantly different among ILO severity groups, with higher fiber-year exposures among groups 3 and 2 compared to group 1 ($p<0.05$). Smoking status also differed significantly across ILO severity groups, with higher ILO severity groups having more ex-smokers and less current smokers ($p=0.0002$). Gender was not

statistically different across ILO severity groups (p=0.1041), but low cell counts may have impacted the validity of the chi squared test.

TABLE VIII - Serum KIF5A, KIF18A, Asbestos Exposure, Age, Gender, and Smoking Status of Asbestosis Patients by ILO Severity Score[a]

	ILO Category 0 (n=9, 11%)	ILO Category 1 (n=43, 52.4%)	ILO Category 2 (n=25, 30.5%)	ILO Category 3 (n=5, 6.1%)	[b]
Age at Sample Collection (years)	55.49 (12.31)	58.91 (7.95)	57.88 (8.17)	55.9 (6.47)	(p=0.0006)*
Gender					(p=0.1041)
Male	6 (66.67%)	37 (86.05%)	23 (92%)	5 (100%)	
Female	1 (11.11%)	5 (11.63%)	1 (4%)	0 (0%)	
Smoking					(p=0.0002)*
Current	2 (22.2%)	24 (55.8%)	8 (32%)	1 (20%)	
Former	3 (33.3%)	10 (23.3%)	11 (44%)	4 (80%)	
Never	2 (22.2%)	8 (18.6%)	5 (20%)	0 (0%)	
Asbestos exposure (years)	25.14 (7.8)	20.26 (9.84)	20.04 (8.07)	21.8 (4.27)	(p=0.0270)*
Asbestos exposure (fiber-years)	624.9 (662.1)	424.6 (477.7)	571.3 (453.8)	737.5 (393.7)	(p<0.0001)*
KIF5A (ng/mL)	4.23 (3.36)	3.4 (2.32)	3.46 (2.72)	4.09 (0.82)	(p=0.1741)
KIF18A (ng/mL)	503.55 (232.56)	390.07 (138.2)	455.21 (185.56)	513.86 (126.88)	(p=0.0014)*
p53AAbs					(p=0.0494)*
Positive	0 (0%)	5 (11.6%)	5 (20%)	1 (20%)	
Negative	9 (100%)	37 (86%)	19 (76%)	4 (80%)	

[a] Age, asbestos exposure (years and fiber-years), KIF5A, and KIF18A are represented as: mean (standard deviation); Gender, smoking, and p53 autoantibody status are represented as: n (%).

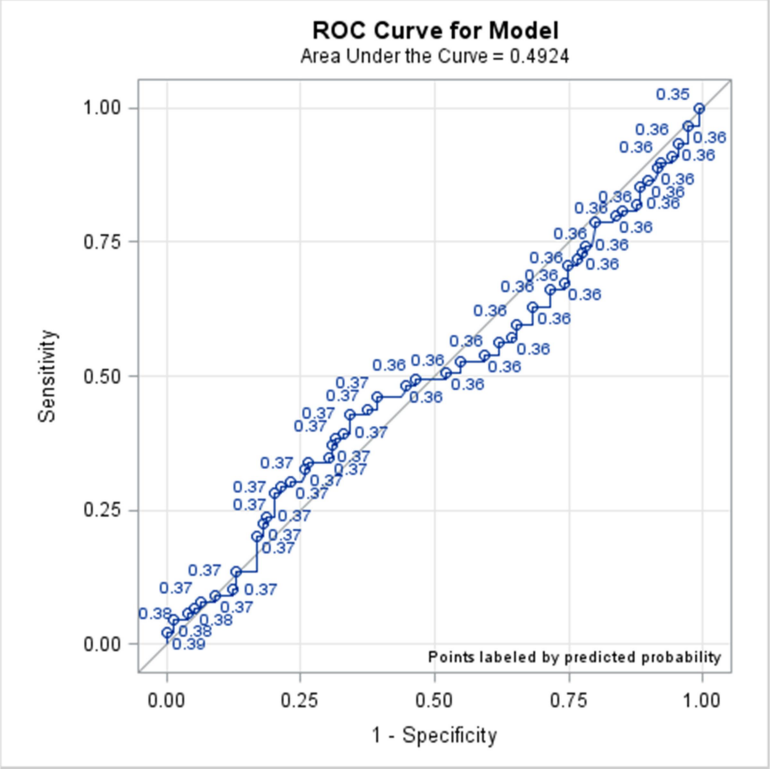
[b] p-values from Wilcoxon rank-sum test for Age, Asbestos exposure (years and fiber-years), KIF5A, KIF18A, and Chi squared test for gender, smoking, and p53 autoantibodies. * indicates significant p<0.05

As reported in separate analyses of this cohort KIF18A was inversely correlated with age (rho= -0.16, p=0.0146) and borderline significantly with exposure (fiber-years) (rho=0.13, p=0.0491); age was directly correlated with exposure years (rho=0.32, p<0.0001), but was inversely associated with fiber-years (rho= -0.29, p<0.0001); and exposure years were significantly correlated with fiber-years (rho=0.42, p<0.0001). Neither KIF5A nor KIF18A were significantly correlated with exposure (years) and only KIF18A was borderline significantly correlated with fiber-years. Additionally, KIF5A levels were significantly lower (p<0.05) in never smokers compared to current smokers or former smokers, but there were no significant differences in KIF18A levels between current, former, or never smokers. There were no significant differences in KIF5A or KIF18A levels between males and females. We found KIF5A levels were significantly higher (p=0.0076) among p53 autoantibody negative individuals, but KIF18A levels were not related to p53 autoantibody status (p=0.6984). p53 autoantibody status was not related to smoking status (p=0.48), but was related to gender (p=0.03) although the validity of the chi squared test may have been impacted by low cell count as there were no positive females. Age was not significantly related to p53 autoantibody status (p=0.0733), but exposure (years) was significantly higher among p53 autoantibody positive individuals (p<0.0001) as was exposure (fiber-years) (p=0.0077).

Separate ROC models were run to assess the predictive performance and optimal cut-point for KIF5A and KIF18A in logistic regression models of ILO major categories 2 and 3 versus categories 0 and 1 utilizing the generalized estimating equations method to account for repeated measures for individuals. The ROC analysis for KIF5A expression, shown in Figure 8, confirmed that it was not significant as a predictor of fibrosis level (p=0.8490) and no cut point

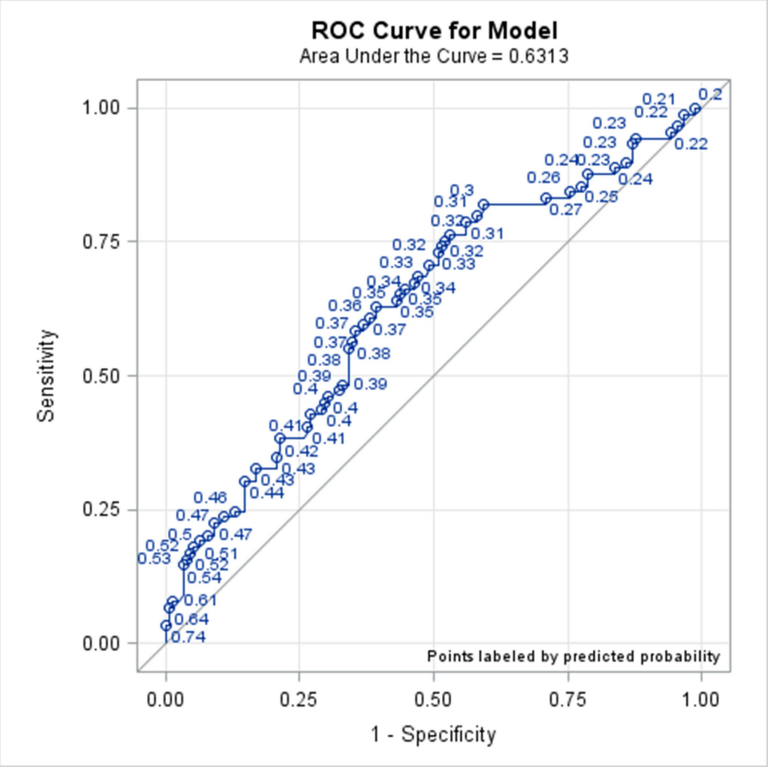
could be determined that adequately distinguished individuals with higher ILO scores from those with lower ILO scores.

Figure 8. KIF5A ROC Curve for ILO Severity Scores



The ROC analysis for KIF18A, shown in Figure 9, was highly significant (p=0.0003) and corresponded to an optimal cutpoint of 431.42 ng/mL using both Youden's statistic and Euclidean distance methods. This cutpoint had a sensitivity of 61.8%, specificity of 61.39%, positive predictive value of 47.41%, and a negative predictive value of 74.05%.

Figure 9. KIF18A ROC Curve for ILO Severity Scores



Multivariable logistic regression models for ILO major categories 2 and 3 versus categories 0 and 1 as well as multinomial regression models were run to assess the potential effects of age, gender, smoking, and asbestos exposure (in years and fiber-years) on the potential relationships between kinesin serum concentrations and/or p53 autoantibody status and ILO severity scores (Table IX). Continuous KIF5A, KIF18A, and p53 autoantibody positive status were not significantly associated with ILO severity score in any model and none of the covariates

were significant in any model. However, continuous KIF18A approached statistical significance (p=0.0794) as did binary KIF18A as defined by the cutpoint determined from ROC modeling (p=0.11) when in a multinomial model for ILO severity scores. Continuous KIF18A also approached statistical significance (p=0.0916) in a multinomial model for ILO severity scores that included p53 autoantibody positive status. In each model increased KIF18A serum concentrations were associated with increased odds of more severe ILO scores (Table IX). While p53 autoantibody status was not significant in any of the final models, positive status showed a consistent association with increased odds of more severe ILO scores. Ex-smoking status also showed a consistent non-significant association with increased odds of more severe ILO scores, likely due to cessation of smoking being a key aspect of clinical recommendations in asbestosis patients.

A sensitivity analysis was done to investigate the potential impact of removing individuals with ILO scores of 0 from the analysis. Continuous KIF18A did become significant in a multinomial model of ILO severity (p=0.0022) with the covariates of current and former smoker status and asbestos fiber-years, none of which were statistically significant. An increase of 100 ng/mL had 1.34 times the odds of more severe ILO scores. In the logistic model of ILO groups 2/3 vs. 1 binary KIF18A was borderline significant (p=0.0682) with only asbestos fiber-years retained in the final model. KIF18A positive status had 1.02 times the odds of more severe ILO scores.

TABLE IX - Final Regression Model Results for Associations of KIF5A, KIF18A, p53 Autoantibodies and ILO Severity Scores[a]

	KIF5A			KIF18A			p53AAbs			Current Smoker			Former Smoker			Asbestos Fiber-years		
	beta-coefficient	p-value	OR (95% CI)	beta-coefficient	p-value	OR (95% CI)	beta-coefficient	p-value	OR (95% CI)	beta-coefficient	p-value	OR (95% CI)	beta-coefficient	p-value	OR (95% CI)	beta-coefficient	p-value	OR (95% CI)
Model 1: Binary ILO	-0.0006	0.5327	1.0 (0.998, 1.001)							-0.4686	0.491	0.63 (0.16, 2.38)	0.73	0.2783	2.08 (0.55, 7.77)	0.0006	0.2314	1.06 (0.96, 1.17)
Model 2: Binary ILO				0	0.3219	1.0 (1.0, 1.0)				-0.469	0.4907	0.63 (0.16, 2.37)	0.7295	0.2788	2.07 (0.55, 7.76)	0.0006	0.232	1.06 (0.96, 1.17)
Model 3: Multinomial	-0.0339	0.6374	0.97 (0.84, 1.11)							0.0039	0.9947	1.0 (0.32, 3.17)	0.9094	0.1883	2.48 (0.64, 9.62)	0.0007	0.171	1.07 (0.97, 1.18)
Model 4: Multinomial				0.0018	0.0794	1.2 (0.98, 1.47)				-0.0405	0.9435	0.96 (0.31, 2.94)	0.885	0.1857	2.42 (0.65, 8.99)	0.0007	0.1692	1.07 (0.97, 1.17)
Model 5: Binary ILO				0.0075	0.1427	1.01 (0.998, 1.02)				-0.4691	0.4906	0.63 (0.16, 2.37)	0.7292	0.2789	2.07 (0.55, 7.76)	0.0006	0.2321	1.06 (0.96, 1.17)
Model 6: Multinomial				0.6586	0.1126	1.93 (0.86, 4.36)				-0.1165	0.8455	0.89 (0.28, 2.87)	0.7736	0.2522	2.17 (0.58, 8.15)	0.0007	0.1666	1.07 (0.97, 1.17)
Model 7: Binary ILO							0.8211	0.1903	2.27 (0.67, 7.77)	-0.3931	0.5774	0.68 (0.17, 2.69)	0.8045	0.2397	2.24 (0.58, 8.55)	0.0006	0.2571	1.06 (0.96, 1.17)
Model 8: Multinomial							0.8486	0.1745	2.34 (0.69, 7.95)	0.0407	0.9461	1.04 (0.32, 3.39)	0.9442	0.1687	2.57 (0.67, 9.86)	0.0006	0.2214	1.06 (0.96, 1.17)
Model 9: Multinomial				0.0017	0.0916	1.19 (0.97, 1.45)	0.7956	0.1748	2.22 (0.70, 6.99)	0.0328	0.9552	1.03 (0.33, 3.24)	0.9734	0.1478	2.64 (0.71, 9.89)	0.0006	0.2373	1.06 (0.96, 1.17)
Model 10: Binary ILO				0.0161	0.1313	1.02 (0.995, 1.04)	0.8222	0.1894	2.28 (0.67, 7.77)	-0.3927	0.5776	0.68 (0.17, 2.69)	0.8048	0.2392	2.24 (0.59, 8.54)	0.0006	0.2579	1.06 (0.96, 1.17)

[a] Final regression model results for Binary ILO score (categories 2/3 vs. 1/0) and Multinomial (ordinal) models of ILO score. KIF5A is continuous in all models; p53AAbs is binary in all models; Models 2, 4, and 9 use continuous KIF18A; Models 5, 6, and 10 use binary KIF18A; both current and former smoker status are binary; asbestos fiber-years are continuous in all models. Odds Ratio (OR) for KIF5A is for a 1 ng/mL increase; OR for KIF18A in Models 2, 4, 9 are for a 100 ng/mL increase and in Models 5, 6, 10 are for positive KIF18A status; OR for p53AAbs are for positive status; OR for current and former smoker status are compared to never smokers; OR for asbestos fiber-years are for a 100 fiber-year increase. All models (n=241) as 6 observations were dropped due to missing values.

6.4 Discussion

In an effort to clarify the potential relationship of altered kinesin expression, p53 autoantibody expression, and asbestos exposure and subsequent asbestos-related diseases this study examined the relationship of the three potential biomarkers to asbestosis severity as defined by ILO International Classification of Radiographs of Pneumoconioses scores. In this analysis we have detected no significant relationship between KIF5A serum concentrations and asbestosis severity. KIF18A serum concentrations distinguished ILO groups (2/3) from ILO groups (0/1) statistically significantly better than chance in ROC modeling. p53 autoantibody status was also significantly associated with ILO scores in bivariate analyses, but the associations was not significant in multivariate regression models. Both continuous KIF18A levels and binary KIF18A status, as defined by ROC analysis, were borderline statistically significant in ordinal models of ILO severity score and binary KIF18A status was borderline significant in a model of binary ILO severity score. In all cases increased serum KIF18A concentrations were associated with increased odds of more severe ILO scoring of asbestosis. We also found a non-significant association of increased odds of higher ILO severity scores in patients that were positive for p53 autoantibodies using both ordinal and binary models of ILO severity scores. Additionally, asbestos exposure was related to ILO severity scores in univariate analysis, but was not significant in any multivariate regression model of ILO severity

In sensitivity analyses where individuals with ILO scores of 0 were dropped the association for continuous KIF18A and ILO scores became highly statistically significant in multinomial modeling and KIF18A positive status remained borderline significant in logistic modeling of ILO

groups 2/3 vs group 1. p53 autoantibody status was not statistically significant and was dropped from sensitivity analysis models. The association between asbestos fiber-years and ILO severity became borderline significant (p=0.0862) in sensitivity analysis multinomial modelling when ILO group 0 was dropped.

There are several limitations to this study including the use of the most severe ILO group score over follow up as representative of that individual's asbestosis severity at all time points. In addition, ten percent of this cohort of diagnosed asbestosis patients was assigned ILO major group scores of 0, which was addressed in sensitivity analysis by dropping group 0, although that further reduced the sample size available for analyses. The size of the Finnish cohort has been reduced by a third due to sample use in previous studies, which likely impacted our ability to detect statistical differences due to the lowered sample numbers. The two kinesin ELISAs had high variability in the serum concentrations found among our study population and had much larger inter-assay coefficients of variation than were reported by the manufacturer. While our inter-assay CVs were calculated from a small number of replicates and would have benefitted from more replicates having been run on each plate, this may reflect some inaccuracy in the ELISA kits or procedure that may have biased our findings either toward finding a relationship that does not exist or failing to find a relationship that does exist between the serum kinesin concentrations and the various cancers. Another potential limitation of our present study was the need to use serum concentrations of the kinesins as a marker, whereas previous studies in the literature examined differences between expression levels in normal and cancer tissues (Shichijo et al., 2005; Liao et al., 2014; Zhang et al., 2010; Nagahara et al., 2011). Additionally, potential sample degradation could have occurred as these samples were collected several

decades before this analysis was conducted and the long-term stability of these kinesin proteins in frozen serum samples is not well characterized. Finally, fiber-years of asbestos exposure were not a significant predictor of ILO severity in any regression model in this analysis. Despite these limitations, this was the first study to examine KIF5A, KIF18A, and p53 autoantibodies and a potential relationship to asbestosis severity in a well characterized cohort of occupationally exposed asbestosis patients.

We have previously shown in an Italian cohort that KIF5A levels are significantly higher in asbestos-exposed individuals without asbestosis compared to unexposed individuals. Additionally we found that KIF18A serum levels are significantly lower in younger and middle-aged individuals, but significantly higher in older individuals who were exposed to asbestos compared to unexposed individuals. These relationships between kinesin expression and asbestos exposure were not confirmed in analysis of this Finnish cohort of asbestosis patients where all individuals had relatively high cumulative asbestos exposure. Additionally, while initial results suggested a potential relationship between KIF5A and KIF18A expression and asbestos-related cancer risk, there was no significant relationship found in follow up analysis of the full Finnish cohort. Lung tissue is one of the few normal tissues where KIF18A is detectable and given our previous findings in the Italian cohort there was an indication that perhaps alterations in KIF18A serum concentrations occurred earlier in response to asbestos exposure and disruption of the lung. Here we have found increased KIF18A expression associated with more severe asbestosis scores. This may reflect that there is an initial decrease in KIF18A serum concentrations following asbestos exposure, but a subsequent increase in KIF18A serum concentrations as severity of asbestosis increases resulting from damage to lung tissue. Further

follow-up and study of the Italian cohort and other asbestos exposed cohorts may help clarify the potential relationship of altered kinesin serum concentrations and asbestos exposure and resultant disease.

7. CONCLUSIONS

Together these studies have examined three potential biomarkers of asbestos exposure and disease in two cohorts of individuals representing the spectrum from unexposed individuals to occupationally exposed individuals without asbestosis, to asbestosis patients with varying severity of disease, to asbestosis patients with asbestos-related and other cancers (Figure 10).

Figure 10. Flow Diagram of Italian and Finnish Cohorts

Alterations to p53 and the formation of p53 autoantibodies have been previously identified in a number of pre-malignant diseases and cancers (Cordes et al., 2009; Anderson et al., 2010; Mattioni et al., 2013; Suppiah and Greenman, 2013; Li et al., 2005). The fact that p53 autoantibodies are detectable in individuals prior to the development or clinical diagnosis of malignant disease with reported lead times to diagnosis ranging anywhere from less than 1 year to 12 years suggests they may possess predictive value for subsequent development of cancer (Li et al., 2005; Mattioni et al., 2013; Pedersen et al., 2013). While previous analysis of this Finnish cohort found p53 autoantibodies were borderline statistically significantly

associated with cumulative asbestos exposure (Li et al., 2005), we found no evidence of a significant association between asbestos exposure and p53 autoantibodies in this Italian cohort. These somewhat discrepant results may be due to the Finnish cohort being composed of asbestos exposed individuals with diagnosed asbestosis as compared to the Italian cohort being composed of asbestos exposed individuals without asbestosis or some other unmeasured difference between the two cohorts. However, the borderline significant finding in the Finnish cohort and the negative finding in the Italian cohort suggest p53 autoantibodies are likely not the strongest biomarker candidate for measuring or representing asbestos exposure. Given the well-established relationship between p53 mutations and p53 autoantibodies and numerous cancers including asbestos-related cancers, our finding of an absence of a relationship to asbestos exposure may also indicate that p53 mutations and subsequent autoantibody formation occur nearer to the development of malignant changes and overt disease.

Additionally, our study of ILO severity found no individuals classified in ILO major category 0 were positive for p53 autoantibody production and increasing percentages of individuals were positive for p53 autoantibody status in higher ILO categories. This would seem to indicate that as the severity of asbestosis increases, the likelihood of p53 autoantibody production increases, strengthening the argument that p53 mutations and autoantibody production are more likely as asbestos-related disease progresses. This finding was limited by low sample size, especially among higher ILO categories, and should be further explored in other asbestosis cohorts for confirmation. It may be important to monitor for p53 autoantibody production among asbestos exposed individuals, especially those with asbestosis, as it may serve as an important marker of worsening severity of disease and of increased risk of asbestos-related cancer development.

The two other potential biomarkers examined in these studies, KIF5A and KIF18A, are members of the kinesin superfamily of proteins, a conserved class of microtubule-dependent molecular motor proteins that support several critical cellular functions such as mitosis, meiosis, and the transport of macromolecules. A growing body of evidence suggests that altered KIF expression and function may play a role in the development and progression of a number of different human cancers, including in the lung (Yu and Feng, 2010). A previous study of a subset of stored serum samples from the Finnish cohort identified three protein peaks that could predict the development of cancer with good sensitivity (0.87) and specificity (0.70), with two of the peaks corresponding to KIF5A and KIF18A (Tooker et al., 2011). In our expanded analysis of the Finnish cohort we found no difference in serum KIF5A or KIF18A levels between any of the observed cancer patient populations and their non-cancer controls. This may be due to differences between the subset of cancer patients and controls selected for the initial screening study, as compared to the broader cohort, or due to the limitations of the original method used to measure and identify the potential biomarkers. It may also be due to the potential influence of the third unidentifiable protein included in the original study's panel.

Our analysis of KIF5A and KIF18A in the Italian cohort did find, however, that KIF5A levels are significantly higher in asbestos-exposed individuals free of asbestosis compared to unexposed controls. We also found that KIF18A serum levels are significantly lower in younger and middle-aged individuals, but significantly higher in older individuals who were exposed to asbestos compared to unexposed individuals. Lung is one of the few normal tissue types where KIF18A is detectable and the alterations in its serum concentrations in asbestos exposed individuals may signal an early disruption to its expression and function in the lung tissue. While

less is known about KIF5A and its potential role in disease, the increased expression of KIF5A seen in asbestos exposed individuals may be an important early marker of molecular changes induced by asbestos exposure resulting in disrupted normal cellular processes. In our analysis of KIF5A and KIF18A and ILO International Classification of Radiographs of Pneumoconioses scores in the Finnish cohort we found no significant relationship between KIF5A serum concentrations and asbestosis severity, but we did find a statistically significant relationship between increased KIF18A serum concentrations and ILO severity scores. Combined with the findings of decreased KIF18A serum concentrations in younger and middle aged individuals, and increased KIF18A serum concentrations in older individuals, associated with asbestos exposure, this may indicate an initial decrease in KIF18A in response to exposure and insult followed by an adaptive response in lung tissues or increased lung cell death leading to the increased serum concentrations seen associated with more advanced asbestosis cases. The increased serum concentrations seen in older exposed individuals may indicate an increased susceptibility to lung damage and cell death that results in an accelerated path to increased serum KIF18A concentrations. Further follow up for development of disease and utility of these kinesins as biomarkers in the Italian cohort as well as other asbestos exposed cohorts may help clarify their role in the exposure-disease pathway.

It seems that p53 autoantibodies would be useful as a screening tool in occupationally exposed asbestos individuals, especially those who have developed asbestosis, to aid in monitoring the severity of asbestosis present and potential risk for asbestos-related cancer development. The statistically significant association found for KIF5A and asbestos exposure should be further explored, especially in occupationally exposed populations where airborne

concentrations of asbestos are available as KIF5A may serve as a useful biomarker of internal dose of exposure. Further work should be done to develop understanding of background levels of KIF5A serum concentrations in the general population so as to help refine an appropriate cutpoint to be used in determining aberrant KIF5A serum concentrations. In occupational populations exposed to asbestos increased KIF5A serum concentrations may be useful in determining if engineering controls and/or personal protective equipment are adequate and being implemented and adhered to appropriately so as to prevent internal doses of exposure from occurring and subsequent disease development. While a statistically significant association was found between KIF18A serum concentrations and asbestos exposure, its modification by age may limit the usefulness of KIF18A as a biomarker of exposure. Additionally, further work should be done to evaluate the potential relationship between KIF18A and ILO severity scores before broader screening is investigated as we found a consistent, but mostly borderline statistically significant association.

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